ANALYSIS OF THE ROLE OF THE *HvMAPK4* GENE IN THE BARLEY BIOTIC STRESS RESPONSE

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ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades play a crucial role in several developmental and physiological processes in plant life, including responses to different biotic and abiotic stresses. Expression of the barley *HvMAPK4* gene is known to be up-regulated after the infection by the blast pathogen *Magnaporthe oryzae*, thus in order to investigate the role of this gene in barley pathogen defence mechanism, different transgenic barley lines have been generated that have either a constitutive expression of *HvMAPK4* or down regulation of *HvMAPK4* by means of an antisense strategy.

Both *HvMAPK4* overexpression and antisense lines were challenged with the blast pathogen, as well as barley wildtype and transgenic line with the empty binary vector (pWBVec.8) as controls. The results showed enhanced disease resistance in antisense lines compared to overexpression lines and controls. The level of resistance in antisense lines was accompanied by an elevated level of endogenous salicylic acid and hydrogen peroxide after infection. Enhanced expression of pathogenesis-related protein (PR1) post-inoculation was seen in these lines along with a significant reduction in catalase activity. The opposite was found in *HvMAPK4* overexpression plants which produced lower amounts of salicylic acid and hydrogen peroxide and showed elevated production of ethylene and an increase in catalase activity in response to the pathogen. The level of jasmonic acid was found to be elevated in these lines, even in the absence of biotic stress.

These results suggest that the barley MAPK (*HvMAPK4*) acts as a negative regulator in barley resistance to the hemibiotrophic pathogen *M. oryzae*, and may act by regulating salicylic acid levels in the plant. *HvMAPK4* is also a positive regulator of jasmonic acid and ethylene production, important compounds in the plant response to wounding and necrotrophic pathogen.

With my love and humility

I dedicate this work to:

Mohammed Al-Khatem, Son

Zahraa, Daughter

Sahar J. Jabbar, Wife

Mohammed, 2011

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DECLARATION

I, Mohammed H. Abass, hereby declare that I am the author of this thesis. All the work described in this thesis is my own, except were stated in the text. The work presented here has not been accepted in any previous applications for a higher degree. All the sources of information have been consulted by myself and are acknowledged by means of reference.

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ABBREVATIONS AND SYMBOLS

° C	degree Celsius
µg	microgramme
µl	microlitre
Amp	Ampicillin
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indoyl phosphate
BLAST	Basic local alignment search tool
bp	base pair
CAT	Catalase
cDNA	copy or complementary DNA
CFU	Colony forming unit
cm	centimetre
cv.	cultivar
dH_2O	distilled water
DIG	Digoxygenin
DMSO	Dimethylesulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside 5´-triphosphate
DEPC	Diethylepyrocarbonate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetraacetic acid
e.g.	Example
ETI	Effector triggered immunity
ETS	Effector triggered susceptibility

FW	Fresh weight
g	gravitational force
H_2O_2	Hydrogen peroxide
h	hour
IE	Immature embryo
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
ISR	Induced systemic resistance
JA	Jasmonic acid
Kan	Kanamycin
kb	Kilo base pair
KD	Kilo Dalton
l	litre
LB	Luria Bertani
LRR	Leucine rich repeat
M	Molar
mA	Milli Ampere
MAPKKK	Mitogen activated protein kinase kinase kinase
MAPKK	Mitogen activated protein kinase kinase
MAPK	Mitogen activated protein kinase
mg	milligramme
min	minute
ml	millilitre
mM	millimolar
mRNA	messenger- RNA
NBT	Nitroblue tetrazolium
ng	nanogramme
OCS	Octopine synthase terminator

OD_{xnm}	Absorption at wavelength of x nm
ORF	Open reading frame
PAMP	Pathogen associated molecule pattern
PCR	Polymerase chain reaction
Pfu	*Pyrococcus furiosus*
pg	picogramme
pI	Isoelectric point
PIPES	1,4-Piperazinediethanesulfonic acid
pmol	picomolar
PR protein	Pathogenesis related protein
PTI	PAMP triggered immunity
RGM	Regeneration medium
RLK	Receptor like kinase
RNA	Ribonucleic acid
RNase	Ribonuclease
SA	Salicylic acid
SAR	Systemic acquired resistance
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SIR	Systemic induced resistance
Taq	*Thermus aquaticus*
Tris	2-Amino-2-hydroxymethylpropane-1.3-diol
UV	Ultraviolet
V	Volt
v/v	volume per volume
w/v	weight per volume
wt	Wildtype

CHAPTER 1 INTRODUCTION

1.1 Economical importance of barley

Barley (*Hordeum vulgare* L.) is the world's fourth most important cereal crop and comes after wheat, maize and rice in terms of production; in 2010 the world production of barley was 124.56 million metric tons, while the cultivated area was 51.14 million hectares throughout the world. In addition, barley is the second most cultivated cereal crop in United Kingdom with 0.9 million hectares grown in 2010 with a total production of 5.20 million metric tons (USDA, 2010). Barley is a successful cereal crop because the growth of barley requires a short season and it is able to grow in poor conditions. In the UK barley is often the dominant cereal crop in the north and east of Britain where the growing conditions are not so suitable for wheat.

Barley is used for several different purposes, and its largest use is for animal feed, secondly in malting, thirdly in many countries around the world, barley is used for human food, including bread and bread-like foods.

1.2 Botanical description of barley

It is believed that barley was one of the first plant species to be domesticated, and many scientists consider that the origin of the wildtype of barley is in Palestine, Jordan, Syria, southern Turkey and Zagros Mountain in Iran (Badr *et al.*, 2000). Here, a wildtype of barley is still to be found (*H. spontaneum*).

Barley belongs to the grass family Poaceae; the cultivars grown in temperate climates can be divided into winter or spring barleys. The cultivation of winter barley (depending on the latitude and climate) normally starts in October and it is harvested in June, while the cultivation of spring barley starts in February and it is harvested in August, these dates can vary according to weather and geographic location as well. Barley morphology consists of a number of stems (three to six tillers and sometimes more depending on growth conditions) produced in a single plant with different sizes starting from 10 cm and sometimes reaching 150 cm in the tallest types of barley.

Single leaves arise at each node of the stem and are borne alternately at each side; the barley leaf consists of the sheath, ligule, auricle and blade. The spike (head or ear) is produced by multiple spikelets attached to the nodes in a zigzag rachis, each spikelet has one flower. The barley plant is a diploid plant, self-pollinating and the barley flower bearing both a male organ (anther) and female organ (ovary) (dioecious) (McGregor, 1993).

1.3 Cereal transformation

1.3.1 Background

Plant transformation is one of the most important tools to improve crop production and overcome the economic losses that are caused by plant pathogens such as fungi, bacteria and viruses in addition to weeds and insects.

The first successful transformations of cereals were achieved in 1988 by mechanical injection of naked DNA into the immature inflorescence of rye, and by direct transfer of the DNA by electroporation into regenerating protoplasts of maize (*Zea mays)*, rice (*Oryzae sativa*) and orchardgrass (*Dactylis glomerata*) (Rhodes, 1988; Zhang and Wu 1988; Horn *et al.* 1988), while the first successful transformation of wheat and barley was performed in the 1990s by Vasil *et al.* (1992) and Wan and Lemaux (1994), respectively.

1.3.2 Gene delivery technologies

For gene delivery into cereals, several technologies were utilised by scientists such as an uptake of free DNA into the protoplast of monocots (rice and maize: Shimamoto *et al.*, 1989; Golovkin *et al.*, 1993), but it was reported that this protocol was limited to a narrow range of monocot genotypes because of the very low regeneration frequencies which were obtained (Vain *et al.*, 1995), thus nowadays this technique is no longer used.

The second protocol for gene delivery is the particle gun bombardment (also known as biolistics), this technology was developed by Sanford (Sanford, 1990). The main principle of this technology is the delivery of naked DNA into intact plant cells, the naked DNA should be coated firstly onto microparticles (such as tungsten or gold) and then by using gunpowder, or a burst of gas such as carbon dioxide, nitrogen or helium with a definite pressure, the particles are forced through the plant cell wall, and into the cell. The biolistic technique has allowed the transfer of exogenous DNA into the nuclear genome of a wide range of monocot plants starting with the first successful biolistic experiments which used embryogenic cell suspensions of maize (Klein *et al.*, 1988), immature embryos of rice, embryogenic callus of wheat and the immature embryos of barley (Cao, 1988; Vasil *et al.*, 1992; Wan and Lemaux, 1994)

Furthermore, several papers revealed the success of electroporation technique to deliver exogenous DNA into different tissues of the plant such as embryogenic callus, immature embryos and germinating seeds (Arencibia *et al.*, 1995; Xu and Li, 1994; Ahokas, 1989). Finally, *Agrobacterium tumefaciens* mediated transformation is one of the most widely applied protocols for dicotyledonous and now also monocotyledonous plants (section 1.3.5 for more details)

1.3.3 Barley cultivars amenable for transformation

The efficiency of barley transformation is dependent on different parameters, one of these parameters is the cultivar used as a donor plant for transformation (Cho *et al.*, 1998). The barley cultivar Golden Promise is still one of the most widely used cultivars employed for stable barley transformation, and this cultivar is amenable for several DNA delivery protocols.

Wan and Lemaux (1994) have used the biolistic method to deliver the DNA to immature embryos of Golden Promise, while Tingay *et al.* (1997) performed the transformation by using *Agrobacterium* into immature embryos of this cultivar, and Holme *et al.* (2006) performed their transformation by using isolated ovules of this cultivar with *Agrobacterium*. In addition to Golden Promise, many other barley cultivars have been used successfully to deliver the genes by different techniques (Table 1.1).

Table 1.1 Barley cultivars amenable to the generation of stable transgenic plants (other than Golden Promise).

Barley cultivar	Method of gene transfer	Target tissue	Reference
Kymppi	*Agrobacterium*-mediated	pollen cultures	(Creissen *et al.*, 1990)
Golena	Biolistic	immature embryos	(Cho *et al.*, 1998)
Harrington	Biolistic	immature embryos	(Zhang *et al.*, 1999)
Clipper	PEG	induced scutellum derived protoplast	(Nobre *et al.*, 2000)
Schooner	Biolistic	immature embryos	(Wang *et al.*, 2001)
Conlon	Biolistic	immature embryos	(Manoharan and Dahleen, 2002)
Sloop	*Agrobacterium*-mediated,	immature embryos	(Murray *et al.*, 2004)
Igri	*Agrobacterium*-mediated	pollen cultures	(Kumlehn *et al.*, 2006)
Tafeno, Helium and Optic	*Agrobacterium*-mediated	immature embryos	(Hensel *et al.*, 2008)
Modified from Goedeke *et al.* (2007)			

1.3.4 Selectable marker genes for plant transformation

Several antibiotics and herbicides have been used in the last two decades for the selection of transgenic plants by positive selection, which allows growth of the transformed plant cells on selectable media; the majority of published research uses kanamycin or hygromycin, or the herbicide phosphinothricin PPT (Sundar and Sakthivel, 2008).

The positive selection system uses a gene encoding an enzyme that confers the resistance to a specific substrate that is toxic to the untransformed plant cells; therefore, these substrates facilitate the differentiation of the transformed plant cells.

The hygromycin phosphotranferase (*hpt* or *hph*) gene is a selectable marker gene that encodes the HPT enzyme, and delivers resistance to the antibiotic hygromycin B (Waldron *et al.*, 1985). The mechanism of hygromycin action is by impairing protein synthesis in mitochondria and chloroplasts, as these organelles contain ribosomes which are susceptible to hygromycin. As a consequence of hygromycin presence in the plant medium, plants undergo chlorosis and inhibition of plant growth (Becker *et al.*, 1992).

Different concentrations of hygromycin have been used successfully for selection purposes in barley transformation (Table 1.2).

Table 1.2 Different hygromycin concentrations (mg/l) used in barley transformation.

Hygromycin concentration(mg/l)	Type of medium	Stage	Target plant tissues	Reference
10-30	*CIM	Callus selection	Immature embryos	(Hagio *et al.*, 1995)
30	**Regeneration	Shoot		
25	CIM	Callus selection	Immature embryos	(Frame *et al.*, 2002)
50	CIM	Callus Selection	Immature embryos	(Holme *et al.*, 2006)
50	Regeneration	Shoot		
50	***Rooting	Root		
50	CIM	Callus selection	Immature embryos	(Shrawat *et al.*, 2007)
10	Rooting	Root		

*CIM: Callus Induction Medium: this type of medium is used for callus production and selection.

**Regeneration medium: this type of medium is used to produce shoot from callus tissues.

***Rooting medium: this medium is used to stimulate root formation.

1.3.5 *Agrobacterium tumefaciens*-mediated transformation of cereal crops.

The molecular improvement of cereal crops, like many other plant species, has been carried out for different purposes such as improvement of the quantity and the quality of plant productivity, or for pest resistance. Development of efficient protocols for genetic transformation is dependent on the methods used for gene transfer, and for many years *Agrobacterium tumefaciens,* as a tool for delivery of transgenes, has been the reliable method of choice for dicot and monocot transformation (Wang *et al.*, 2001; Table 1.3).

Agrobacterium is a remarkable microorganism and the only known prokaryotic organism that transfers DNA to eukaryotic cells (Bundock *et al.*, 1995). *Agrobacterium*-mediated transfer of genes is a biological process with many advantages over other methods of transformation, as *Agrobacterium* efficiently integrates a small number of copies of large segments of DNA with defined ends into plant chromosomes with little rearrangement (Gelvin, 2003).

Regarding the *Agrobacterium* host range, *Agrobacterium* strains infect a wide range of plants from angiosperms to gymnosperms (de Groot *et al.*, 1998). *Agrobacterium* naturally infects plants through wounds. The chemical compounds which are released from wounded tissues such as phenolic and sugar compounds play a pivotal role in eliciting chemotaxis of *Agrobacterium* cells to the wounded sites of plant tissues (Ashby *et al.*, 1988). The sensing of these compounds is achieved by an *Agrobacterium* signal transduction system via *VirA* and *VirG* which considered as a sensing machinery, *VirA* is acting as a membrane-bound sensor, while the *VirG* is the cytoplasmic regulator (Wolanin *et al.*, 2002). The attachment of *Agrobacterium* to the wounded plant cells is facilitated by the role of different sets of bacterial chromosomal genes including *chvA*, *chvB* and *psc* (McCullen and Binns, 2006).

After the activation of *VirA*, the histidine kinase (HK) of *VirA* will phosphorylate the *VirG* at a particular aspartate (Brencic and Winans, 2005). A different set of *Vir* genes will be activated to generate a mobile copy of T-DNA such as the *VirD1* and *VirD2* proteins which cut the T-DNA from both left and right borders (25-bp direct repeat) and produce a single-stranded T-DNA molecule. One molecule of *VirD*2 will attached the 5' end of the single stranded T-DNA, and then many molecules of *VirE*2 will coated the single strand T-DNA to form a T-DNA complex (Howard *et al.*, 1990). The T-DNA complex then transferred into the host cell through a T-pilus channel formed by *VirD4* and *VirB* proteins (Sheng and Citovsky, 1996, Fig. 1.1).

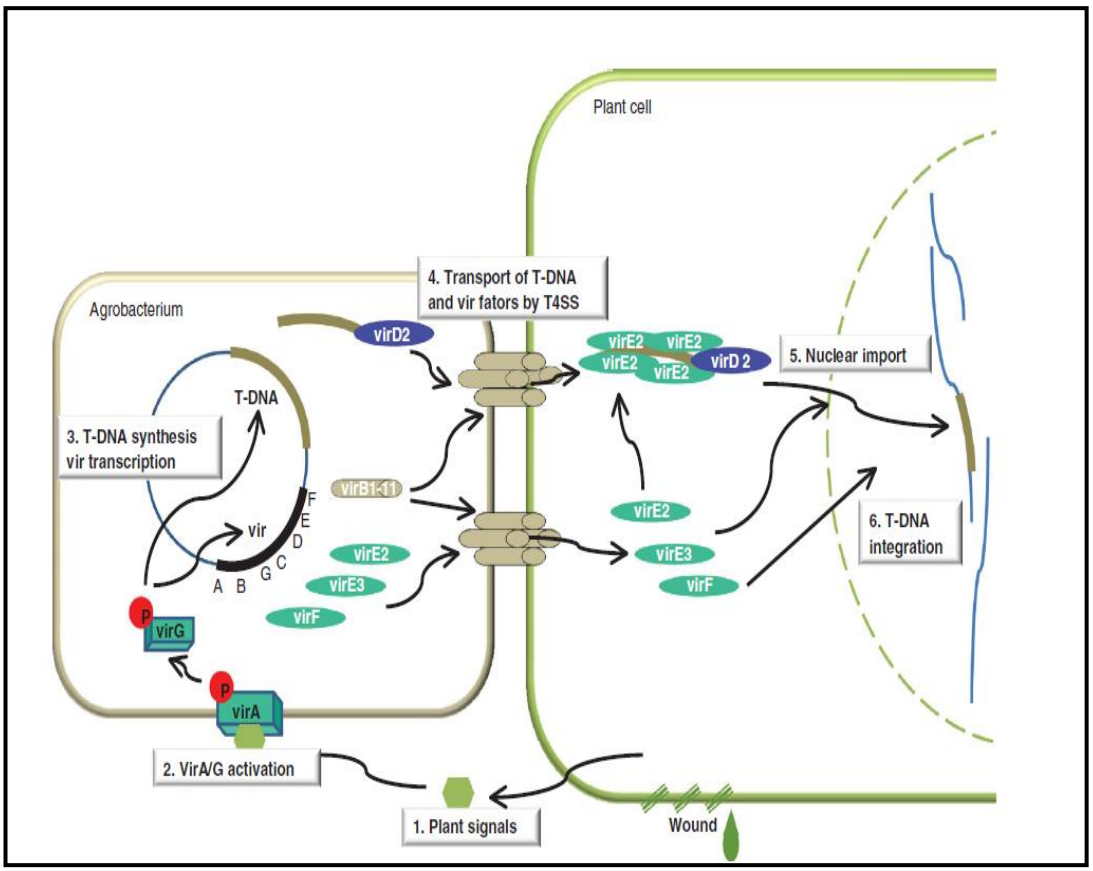


Figure 1.1 *Agrobacterium* transformation process.

1-Plant signals

2-Activation of VirA/G genes.

3-T-DNA synthesis and *vir* genes expression.

4-T-DNA/vir protein complex.

5- T-DNA transporting into the host nucleus.

6-T-DNA integration into the host chromosome.

From Pitzschke and Hirt (2010)

Monocotyledonous plants are not the natural host of *Agrobacterium*, thus, the transformation for these plants via *Agrobacterium* is generally difficult, but this obstacle has been overcome by using super-binary vectors. Ishida *et al.* (1996) used the super binary vector pTOK233, which include an extra copy of the *VirB, VirC* and *VirG* genes, to infect immature embryos of maize. *Agrobacterium* was even reported to transfer DNA to human cells (Kunik *et al.*, 2001).

The transformation efficiency of *Agrobacterium* depends on many factors such as plant species, the plant tissue, antibiotics, the strain of *Agrobacterium*, and co-cultivation methods. Several supervirulent *Agrobacterium* strains such as EHA101, EHA105 and AGL1 have been used in plant transformations (Hellens *et al.*, 2000; Table 1.3). The supervirulent strain *Agrobacterium* EHA105 contains the Ti plasmid pTiBoS42 which has additional *VirG* genes (Jin *et al.*, 1987), and is used in the work presented here.

Different methods have been developed to improve the transformation efficiency of *Agrobacterium* and to overcome host specificity, such as sonication, addition of antioxidant such as polyvinylpyrrolidone, L-cysteine and dithiothreitol, and the addition of acetosyringone which is a phenolic compound used widely to induce the expression of *vir* genes (Perl *et al.*, 1996; Frame *et al.*, 2002).

Table 1.3 *A. tumefaciens*-mediated transformation to different cereal crops.

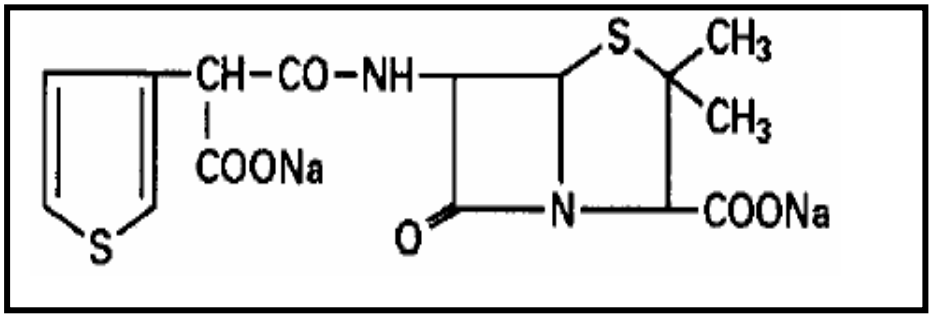
Plant	Cereal cultivars	*Agrobacterium* strain	Reference
Maize	A188	LBA4404	(Ishida *et al.*, 1996)
Rice	Taipei 309	LBA4404-AGL1	(Wang *et al.*, 1997)
Barley	Golden Promise	AGL1	(Tingay *et al.*, 1997)
Wheat	Bobwhite	C58(ABI)	(Cheng *et al.*, 1997)
Wheat	Shiranekomugi	LBA4404	(Supartana *et al*., 2006)
Barley	Golden Promise	AGL1	(Bartlett *et al.*, 2008)
Barley	Golden Promise	LBA4404	(Hensel *et al.*, 2008)
Rice	Hokkaisiryo	EHA 101	(Ozawa, 2009)

The elimination of *Agrobacterium* growth after co-cultivation is a critical step in plant transformation to ensure the successful regeneration of plants through tissue culture course (Nauerby *et al.,* 1997), and this can be achieved by applying the appropriate levels of antibiotics in the regeneration medium thus avoiding any side effect on plant regeneration. Different types of antibiotics used for this depend on the *Agrobacterium* strain and include cefotaxime and carbenicillin, but the literature shows that carbencillin has a negative effect on the regeneration of many plants such as *Arabidopsis, Daucus carota, Solanum tuberosum* and *Triticum aestivum* at the concentration of 500 mg/l which is required to eliminate *Agrobacterium* (Okklels and Pedersen, 1988; Patton and Meinke, 1988; Simmonds and Grainger, 1993; Frederiksen, 1994). Thus, Timentin is now used to prevent *Agrobacterium* growth.

Timentin is a mixture of two components, one is an antibiotic which is carcillin, (a penicillin derivative), and the other component is clavulanic acid which is a beta-lactamase inhibitor, this chemical is widely used to inhibit beta-lactamase in different bacteria resistant to penicillin (Fig. 1.2) Timentin has a strong activity against gram negative bacteria, and the mechanism of action is by binding to the penicillin-binding proteins in the periplasm of the bacteria and interrupting the peptidoglycan biosynthesis thus leading to the death of the sensitive bacteria by cell wall lysis (Nauerby *et al.,* 1997).

Nauerby *et al.* (1997) have also shown that Timentin at 75 mg/l was not sufficient to restrict the growth of *Agrobacterium* on solid media, however the concentration of 150 mg/l was able to completely inhibit growth, with no effect on the regeneration of tobacco shoots and root systems, thus they recommended using Timentin instead of other antibiotics such as carbencillin and cefotaxime in plant tissue cultures. A range of Timentin concentrations (100-150 mg/l) have been used widely in the regeneration of transformed barley callus and embryos after *Agrobacterium*-mediated transformation (Tingay *et al.*, 1997; Wang *et al.*, 1997; Wang *et al.*, 2001; Bartlett *et al.*, 2008; Hensel *et al.*, 2008).

A



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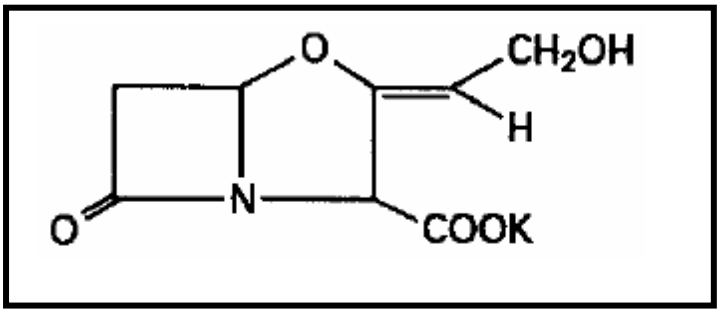


Figure 1.2 Chemical components that comprise Timentin:

A. Tricarcillin.

B. Clavulanic acid.

1.4 Blast disease caused by *Magnaporthe oryzae*

1.4.1 Background

In the natural environment the interaction between plants and various microbes can result in plant diseases, which in turn cause considerable economic damage to modern agriculture. In this attack on the plant, leaves, roots, stems, flowers, fruits and grains of the plant are the target for different plant pathogens, the pathogens obtain their nutrients such as carbohydrates from these organelles and may lead to appearance of severe disease symptoms and in some cases to the death of the infected plant.

The ascomycete fungal pathogen *Magnaporthe oryzae* (Herbert) Barr. (previously known as *M. grisea*, anamorph *Pyricularia oryzae*) is the causal agent of blast disease on different field crops, mainly rice, and there are many strains of this pathogen that have been confirmed to attack a wide range of monocot plants such as barley, wheat and ryegrass (Ribot *et al.*, 2008; Zellerhoff *et al.*, 2006; Kusaba *et al.*, 2006). In addition, this pathogen has been found to attack the *Arabidopsis* plant (Park *et al.*, 2009). This wide host range makes *M. oryzae* a serious plant pathogen for cereal crop production around the world. This fungus is also considered as a model hemibiotrophic fungus for investigating plant-microbe interaction (Gilbert *et al.*, 2006).

1.4.2 The life cycle of *M. oryzae*

The life cycle of this pathogen on the host plant starts when a conidium of *M. oryzae* contacts the cuticle of the host leaf in the presence of free water. This leads to the breakage of the conidial wall (at the apex) and an adhesive drop is released to facilitate conidial attachment onto the plant hydrophobic surfaces. Later, germination of the conidium occurs followed by hyphal growth along the cuticle of the cell wall, and then the appressorium is formed, first as a hook form and in the availability of suitable conditions, these hooks will form appressoria into which most of germinated conidium cell content will be translocated. In this stage of infection, the plant cell is exposed to high turgor pressure as a mechanical force for pathogen invasion, with little involvement of cell wall degrading enzyme (Skamnioti and Gurr, 2007).

After the penetration of the host cuticle and underlying cell wall, the infection hyphae of *M. oryzae* gives rise to branched bulbous hypha which colonize the epidermal cells, then the bulbous hypha produces thinner invasive hyphae which break out of the initial infected cells, and tissue necrosis will start and the development of symptoms will be obvious. Following this, a conidiophore will emerge on the surface of the infected leaf and the conidia start to spread and infect new plants. In favorable conditions the pathogen produces conidia repeatedly for up to 20 days. The pathogen is able to survive in winter on the residue of infected plants or seeds, and weeds can be an alternative host for the blast pathogen. The blast fungus is able to infect all the organs of the host plant (Thines *et al.*, 2004; Skamnioti and Gurr 2009, Fig. 1.3). Recently, Marcel *et al.* (2010) were provided an initial evidence about the ability of the blast pathogen to infect rice root.

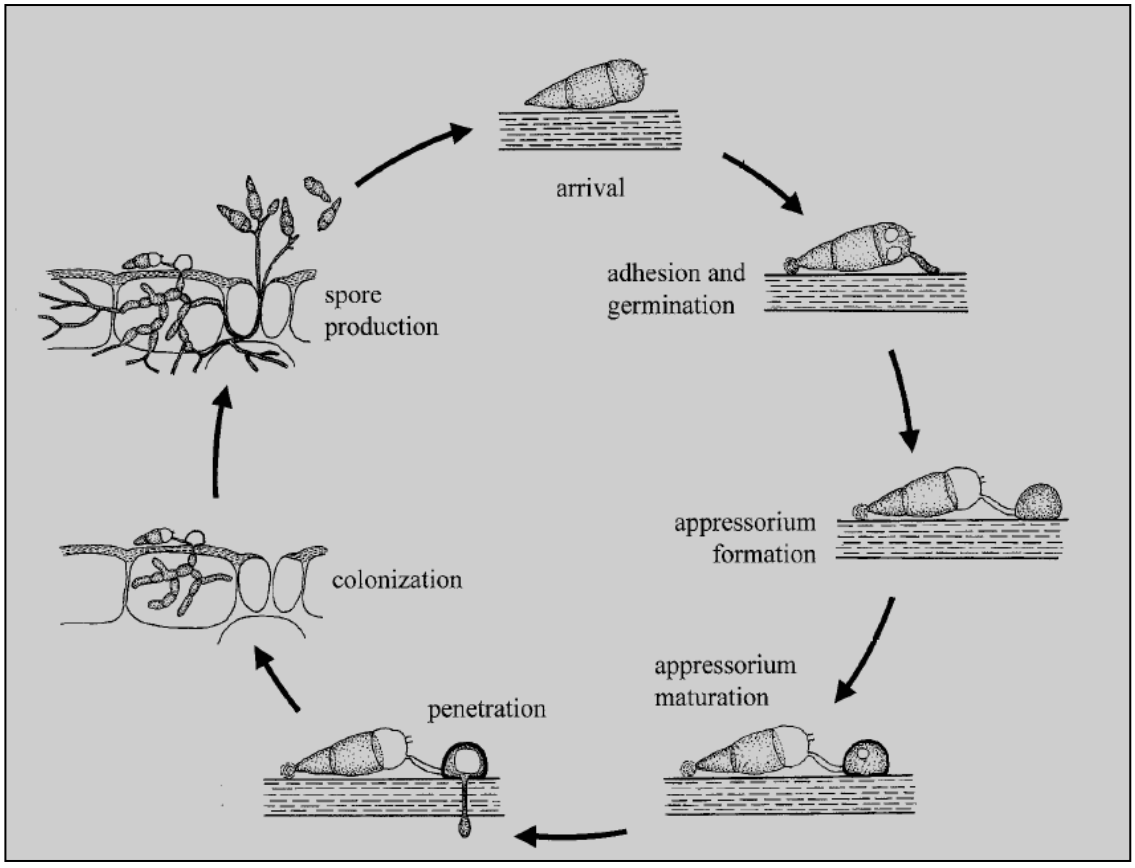


Figure 1.3 The disease cycle of the blast fungal pathogen *M. oryzae*.

From: Thines *et al.* (2004).

1.5 Plant disease resistance

1.5.1 Background

Plants depend on their innate basal immune system to restrict the growth of potential pathogens. These pathogens find a way to invade the plant tissues through different means, such as penetrating the leaf, stem and root system, also, through the wounds or via natural openings such as stomata, hydathodes and lenticels. The plant cell wall is complex and forms a physical barrier toward the pathogen attack, but the plant pathogens have developed different strategies to penetrate the plant cell such as using a cell wall degrading enzyme and cell wall permeable toxins (Dangl and Jones, 2001).

Plants have developed mechanisms to perceive microbial attack and respond with adaptive responses, which lead to disease resistance. The plants have different defence systems, one of these systems is a passive defence system in which preformed barriers or endogenous toxic compounds inhibit the invasion by pathogens. Upon recognition of a possible pathogen, an active defence system is brought into play; this is basal resistance which is triggered about 10-30 minutes after the contact with the pathogen (Ellis *et al.*, 2000; Dangl and Jones, 2001; Jones and Dangl, 2006; Hofius *et al.*, 2007).

There are different lifestyles of plant pathogens which influence the means of achieving disease resistance in the plant. In the biotrophic lifestyle, these types of pathogens cannot survive on dead tissues, thus they are trying to keep their host cells alive and decrease the damage on the cell level and generally show a restricted host range, such as rust and mildew fungal pathogens, bacterial species of *Pseudomonas* spp. and viral pathogens. In contrast, necrotrophic lifestyle pathogens can be differentiated by their ability to kill their host cells by various means, such as producing toxins or cell wall degrading enzymes. These types of pathogens show a wide range of hosts and include the *Botrytis* fungus (a grey mould pathogen) and the rot bacterium *Erwinia* spp. Hemibiotrophic lifestyle pathogens combines both the above mentioned lifestyles and starts their plant invasion with biotrophic behavior, followed by necrotrophic host death, such as the oomycete *Phytophthora infestans* and the rice blast fungal pathogen *Magnaporthe oryzae* (Gurr and Rushton, 2005).

Regarding the plant defence response, there are different systemic responses to pathogen attacks, such as systemic acquired resistance (SAR), which is normally activated by local fungal, bacterial and viral infection. These local infections trigger the local and systemic synthesis of salicylic acid (SA), which drives the synthesis of pathogenesis-related proteins (PR) in distal tissues and the release of methyl-SA.

The systemic induced resistance response (SIR, which is also known as the systemic proteinase inhibitor or wound response) is triggered by mechanical wounding or chewing feeders and leads to an increase in the level of jasmonic acid (JA) and ethylene (ET), and accumulation of systemic proteinase inhibitors and wound response proteins. The third type of systemic response is induced systemic resistance (ISR), which results from non-pathogenic rhizosphere bacteria and involves the transient of JA, ET, and the transient activation of defence responses in distal tissues (Agrios, 1997).

Regarding the systemic acquired resistance which is effective mechanism against hemibiotrophic and biotrophic pathogens, many instances have been published in different plant and pathogen interactions such as rice plants and the hemibiotrophic pathogen *M. oryzae* (Yang *et al.*, 2004). Another example for plant systemic acquired resistance was reported in soybean defence response to the biotrophic rust pathogen *Phakospora pachrhizi* (Srivastava *et al.*, 2011). Whereas, the systemic induced resistance is characterized by the plant response to the necrotrophic pathogens, such as *Arabidopsis* response to different necrotrophic pathogens including *A. brassicicola* and *B. cinerea* (Thomma *et. al.*, 1998), and to plant herbivores such as induced defence response of rice plants to the plant brownhopper (*Nilaparvata lugens*) (Senthi-Nathan *et al.*, 2009).

Regarding the induced systemic resistance**,** the plant growth promoting rhizobacteria (PGPR) *Pseudomonas flourescens* was able to induce systemic resistance in rice plants against rice sheath blight pathogen (*Rhizoctonia solani*), and promote the growth of rice plants (Nandakumar *et al.*, 2001).

1.5.2 Detection of microbes in plant-microbe interaction

Plants have the ability to recognize potential pathogens by the detection of pathogen-associated molecular patterns (PAMPs) and this can be achieved by extracellular receptor-like kinases (RLKs) (Zhao and Qi, 2006, Fig. 1.3). Many conserved microbial components can be recognized by plants such as the structural component of bacteria flagellin, toxins, cold shock proteins, elongation factor Tu (EF-Tu), as well as non-protein PAMPs including bacterial lipopolysaccharides, chitin and ergosterol from higher fungi, *β*-heptaglucosan from *Phytophthora megasperma* and many other oomycetes (Garcia-Brugger *et al.*, 2006). In addition, there is evidence that the induced systemic resistance (ISR), triggered by plant growth-promoting rhizobacteria, is related to PAMP perception (Gomez-Gomez and Boller, 2000).

Plants perceive PAMPs via RLK and this induces PAMP-triggered immunity (PTI). As an example of PTI, it was found that a synthetic 22-amino acid peptide of flg22 from a conserved bacterial flagellin domain is able to induce different cellular responses in *Arabidopsis* such as the rapid transcriptional induction of 1100 genes (Gomez-Gomez and Boller, 2000).

The PTI process requires signalling through mitogen-activated protein kinase (MAPK) cascades and the transcriptional reprogramming of plant gene expression mediated by plant-specific *WRKY* transcription factors. The first identification of WRKY proteins was reported in sweet potato and wild oat (Ishiguro and Nakamura 1994, Rushton *et al.* 1995, Dong *et al.*, 2003). WRKY proteins consist of a super family of transcription factors, found in higher plants. In *Arabidopsis* there are 74 members of the WRKY transcription factor family, and there are 105 members in rice (Dong *et al.*, 2003), whilst in barley about 45 WRKY proteins have been reported (Mangelsen *et al.*, 2008).

Liu *et al.* (2007) investigated the expression of ten *WRKY* transcription factors in rice plants after treatment with SA and JA, their results showed that the *OsWRKY71* gene was induced by both signaling molecules, and they found that the overexpression of this transcription factor gene enhanced the resistance to bacterial blight pathogen in transgenic rice, and activated several defence-responsive genes such as *OsNPR1* and *OsPR-1* in these plants.

WRKY proteins play a regulatory role in a wide range of biotic and abiotic stresses, including pathogen infection (Asai *et al*., 2002), wounding (Hara *et al.*, 2000), drought, cold, and high salinity (Seki *et al.*, 2002). In addition there are several papers that focus on their functions in embryogenesis, senescence, carbohydrate anabolism and secondary metabolism (Hinderhofer and Zentgraf, 2001; Sun *et al.*, 2003; Lagacé and Matton, 2004). The common feature of the WRKY proteins is the WRKY domain with a 60 amino acid region that contains a highly conserved amino acid motif, WRKY-GQK with either a Cys_2-His_2 or Cys-His_2-Cys motif at its N-terminus and a metal chelating Zinc-finger signature at N–terminus (Mangelsen *et al.*, 2008). WRKY proteins regulate the expression of target genes by specifically binding to the (T) TGACC (A/T) {W box} DNA sequence in the promoter region and thus mediates the response to a pathogen elicitor at the transcriptional level (Qiu *et al.*, 2004).

Several studies on barley WRKY proteins have investigated their biological functions, and revealed that the barley *HvWRKY1* is involved in different regulatory processes, such as the response to cold and drought (Marè *et al.*, 2004), while the *HvWRKY2* protein has been involved in the repression of PAMP-triggered basal defence during the infection with *B. graminis* (Shen *et al.*, 2007).

1.5.3 Effectors in plant pathogenic microbes

Successful plant pathogens produce different types of effectors (suppressors) to restrain the basal immune system (this type of interaction is known as effector triggered susceptibility: ETS) and this will lead to increase the colonization and infection (Fig. 1.4). These effector proteins (the recognized effector is termed an avirulence protein, Avr) have the capability of modifying host defence proteins so as to enhance the pathogen´s virulence and evade detection, for example phytopathogenic bacteria such as *P. syringae* can deliver approximately 20-30 different effectors via a type 3 secretion system (TTSS) during the infection processes (Petnicki-Ocwieja *et al.*, 2002).

In the earliest studies on the mechanism of bacterial effector-plant interaction, Jakobek *et al.* (1993) revealed that the bean pathogen *P. syringae* pv. *phaseolicola* (Pph) had the ability to suppress the induction of defence genes in bean. Regarding fungal effectors little is known about their role in pathogenecity (Hofius *et al.*, 2007). The flax rust fungus *Melampsora lini* and the leaf mould pathogen *Cladosporium fulvum* have the ability to produce effectors in the pathogenesis process (Rivas and Thomas, 2005; Catanzariti *et al.*, 2006), and the common feature of these fungal effectors is that they are cysteine-rich and less than 300 amino acid residues in size (Zhao and Qi, 2008). Recently, different *Avr* genes have been identified in the blast pathogen (*M. oryzae*) such as *AVR-Piz-t*, *AVR-Pita1*, *AVR-Pii* and *AVR-Pik/km/kp* which play significant roles in pathogenicity (Yoshida *et al.*, 2009).

The plant recognizes the pathogen effectors by means of plant *R*-genes and this perception triggers the defence response which is known as effector triggered immunity (ETI), which stimulates the hypersensitive reaction (HR) and leads to localized cell death (Chisholm *et al.*, 2006). Each *R* gene confers a vertical resistance (single dominant resistance) to restrict the attack of the pathogen. One of the earliest analyses of *R* genes is the "gene-for-gene" hypothesis, which stated for each *R* gene in plant (host), there is a corresponding gene in the pathogen, which interacts specifically (Flor, 1955).

Five different classes of *R* genes have been identified; the first class is represented by cytoplasmic receptor like proteins which contain leucine rich repeat (LRR) domain and nucleotide binding protein sites (NBS) including *RPS2* and *RPM1* from *Arabidopsis*, *Prf* from tomatoes, *N* from tobacco, L^6 and *M* from flax and I_2 from tomato. The second class of *R* genes encodes a serine-threonine kinase such as *Pto* in tomatoes, while the third class is represented by *Cf-2* and *Cf-9* from tomatoes, and the last class is represented by the maize *HM1* gene which is a toxin reductase (Baker *et al.*, 1997).

Regarding the hypersensitive reaction, this is a rapid host response occurring after the infection by pathogen in a host cell (Lam, 2004); the infected cells die as a response to the infection. HR is a type of active programmed cell death (apoptosis), and this process is characterised by different steps such as chromatin condensation, DNA cleavage and membrane blebbing which leads to membrane disruption and release of cell content (Dat *et al.*, 2003). HR is efficient in restricting the growth of biotrophic pathogen which requires a living cell to feed off; however for necrotrophic pathogens, the HR will be beneficial for the infection and leading to increase the colonisation (Greenberg and Yao, 2004).

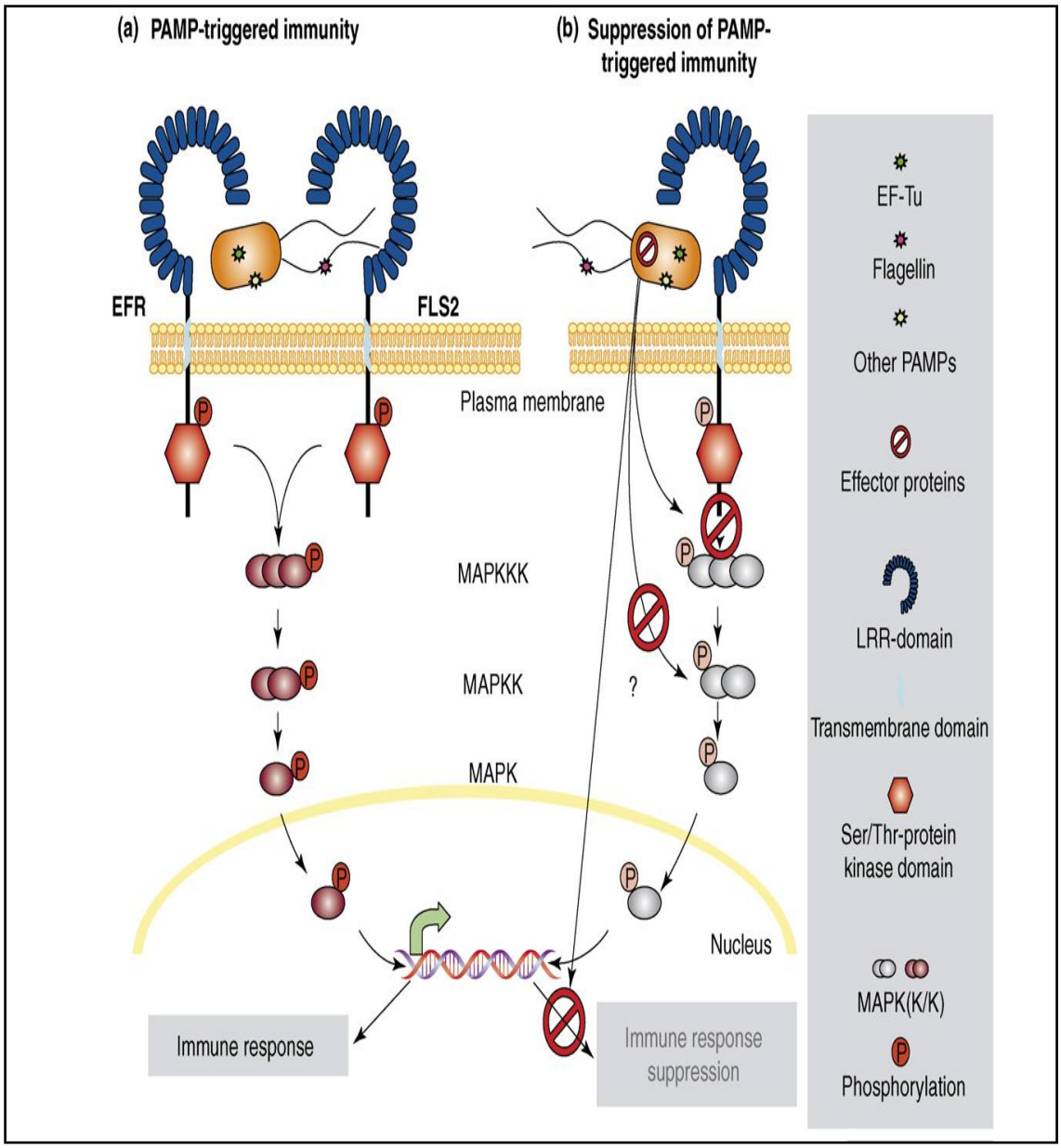


Figure 1.4 Pathogen-associated molecular patterns (PAMPs) induce plant immunity and suppression of PAMP-induced plant immune responses. Leucine-rich repeat (LRR) Receptor Protein Kinases: EFR and FLS2.

From: Nurnberger and Kemmerling (2006)

1.5.4 Pathogenesis-related proteins

The pathogenesis-related proteins can be defined as host-specific proteins which are induced in different species of the plants during the pathogen attack or similar situations (van Loon *et al.,* 2006). The accumulation of PR proteins can result from a pathogen attack such as viral, bacterial and fungal pathogens, nematodes, insects and herbivores. These proteins are absent, or present at low concentrations in healthy tissues, but these concentrations will increase significantly within a short time after infection with pathogens (van Loon and van Strien, 1989).

The first discovery of inducible defence-related proteins was found in tobacco reacting by hypersensitivity to tobacco mosaic virus (TMV) in 1970. Two different groups showed that these proteins are induced in resistant tobacco plants and accompany a hypersensitive necrotic response to viral pathogen (Gianinazzi, 1970; van Loon and van Kammen, 1970). There are now about 17 families of PR proteins that have been identified in different plants, differing in amino acid sequences, serological relationship and biological activity (Table 1.4). Homologues of most of these PR proteins families are to be found in both monocots and dicots.

PR proteins are of low molecular weight (about 10-40 KDa) and they are localized in different locations in the plant such as vacuoles, cell wall and the intercellular space, also, most PRs tend to have extreme isoelectric points (pI) (Stintzi *et al.,* 1993).

Table 1.4 Pathogenesis-related proteins in barley plant.

Family	Class	Type number
PR-1	Antifungal	Tobacco PR-1a
PR-2	Glucanase	Tobacco PR-2
PR-3	Chitinase I and II	Tobacco P, Q
PR-4	Chitin binding, hevein	Tobacco 'R'
PR-5	Thuamatin-like	Tobacco S
PR-6	Thuamatin-like	Tomato Inhibitor I
PR-7	Endoproteinase	Tomato P69
PR-8	Chitinase III	Cucumber chitinase
PR-9	Peroxidase	Tobacco 'lignin-forming peroxidase'
PR-10	Ribonuclease-like	Parsley 'PR1'
PR-11	Chitinase	Tobacco 'class V' chitinase
PR-12	Defensis	Radish Rs-AFP3
PR-13	Thionin	Arabidopsis THI2.1
PR-14	Lipid-transfer protein	Barley LTP4
PR-15	Oxalate oxidase	Barley OxOa (germin)
PR-16	Oxalate oxidase-like	Barley OxOLP
PR-17	Aminopeptidase-like	Tobacco PRp27
From: van Loon et al. (1994)		

The biological functions of PRs are not fully understood for all of the members of the PR families, but PRs can be induced systemically by infection with different pathogens, and certain PRs play a pivotal role in plant defence as proteins that restrict the multiplication and spread of the pathogens (van Loon *et al.*, 2006). Apart from the defence induction, PRs also appear to be multifunctional proteins and many PRs have also been detected during plant development and senescence (Liljeroth *et al.*, 2005).

The PR-1 family is considered as one of the most important PR families, and it was found to be induced by pathogens and salicylic acid, and is commonly used as a marker for systemic acquired resistance (van Loon and van Strien, 1989). The function of the PR-1 family is still not fully clear, but many researchers have attempted to clarify the function of PR-1 in plants (Buchel and Linthorst, 1999). There is much evidence indicating an association between PR-1 proteins and enhanced disease resistance against oomycete and bacterial pathogens. Tobacco plants which constitutively expressed PR-1 showed a resistance against *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotinae* (both of them are oomycete pathogens) (Alexander *et al.*, 1993). In tomato, PR-1 was found to reduce the germination of sporangia and germ tube length of *P. infestans*, in *in vitro* experiments, and this protein was also found to reduce the surface area showing symptoms of tomatoes leaf discs infected with this pathogen when applied *in vivo* (Niderman *et al.*, 1995).

In the paper of Schultheiss *et al.* (2003), it was reported that mutant lines of barley in which PR-1 proteins were lacking or non-functional, allowed the mildew fungus *Blumeria graminis* f.sp. *hordei* to penetrate the barley cell wall more frequently, while Sarowar *et al.* (2005) revealed that overexpressing the TMV-inducible PR-1 encoding gene in tobacco led to enhanced tolerance toward *P. parasitica* var. *nicotinae*, *Ralstonia solanacerum* and *P. syringae* pv. *tabaci*.

1.6 The role of MAP kinases in plant diseases control

The mitogen-activated protein kinase (MAPK) signalling network is a crucial component of intracellular plant communication and regulation, which is initiated from the plasma membrane by extracellular stimuli such as biotic and abiotic stresses (Agrawal *et al.*, 2003a). Protein kinase activation is conserved as one of the most common mechanisms of signal transduction in many cellular processes (You *et al.*, 2007). The basic structure of the MAPK signal transduction pathway is conserved in eukaryotes and consists of three types of kinases: MAPKKK which can be defined as the initiator of signalling, MAPKK which can be defined as the signalling carrier and MAPK which can be defined as the signal carrier into intracellular targets (Liu and Xue, 2007).

MAPKKKs carry specific motifs in their sequences that selectively confer their activation in response to different stimuli, MAPKKK are the first component of this phosphorylation cascade that activates MAPKK by phosphorylating conserved serine/ threonine residues, while MAPKK activates the MAP kinases. The activation of MAP kinase enzyme activity occurs by dual phosphorylation of a tripeptide motif {Thr-Xaa-Tyr} which is located in the activation loop (T-loop) (Hamel *et al.*, 2006).

Plant cells receive many different kinds of stimuli from their environment; therefore, the MAPK phosphorylation system serves as a link in various ways between upstream receptors and downstream targets. (Fig. 1.5)

In plants, many different MAPKs have been characterized and identified from different plant species such as *Arabidopsis*, maize, tobacco, *Petunia*, oat, wheat, barley and rice (Takezawa, 1999; Wilson *et al.*, 1995; Song and Goodman, 2002; Asai *et al.*, 2002; Decroocq-Ferrant *et al.*, 1995; Huttly and Phillips, 1995; Knetsch *et al.*, 1996)

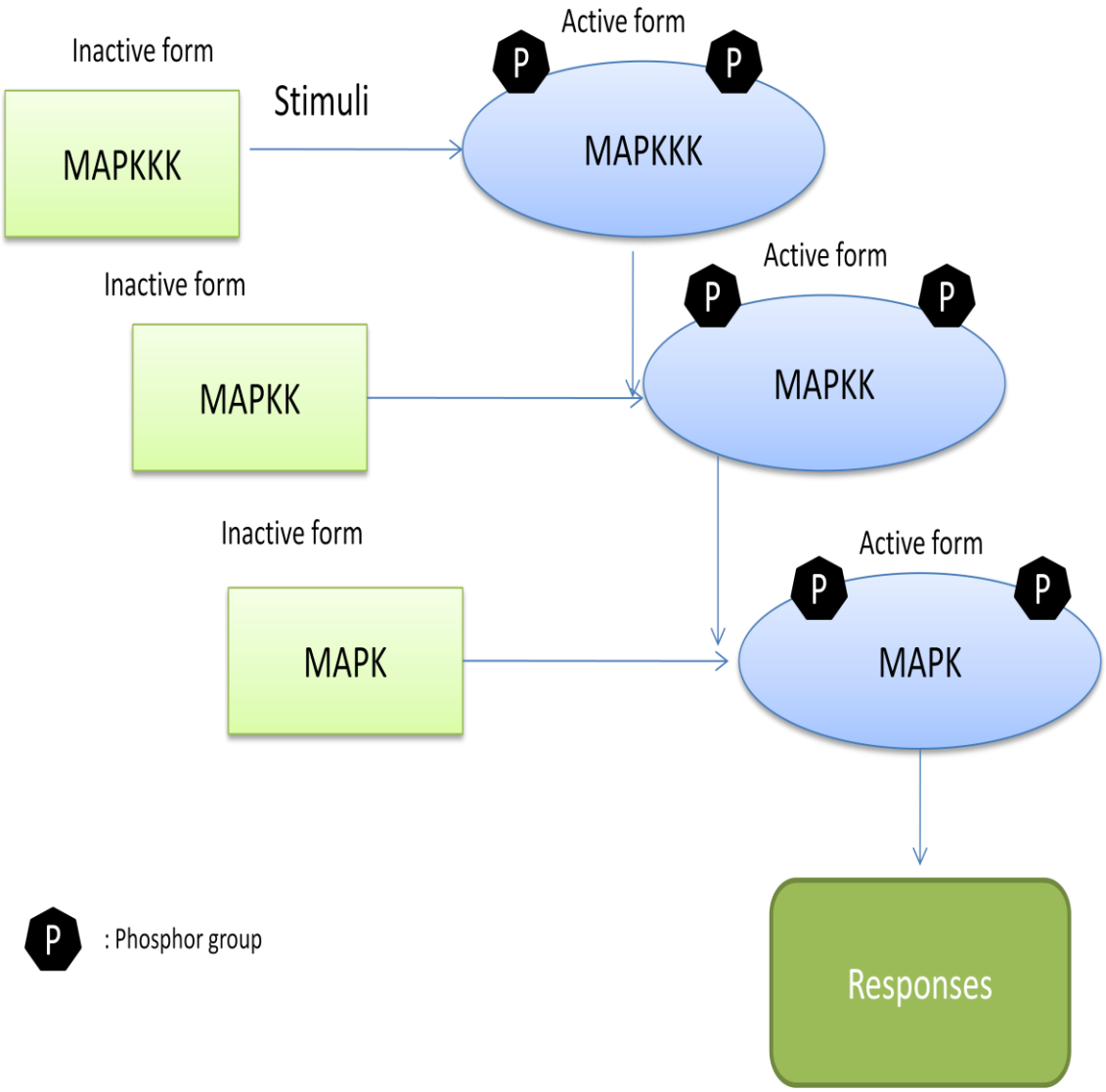


Figure 1.5 Plant MAP kinase phosphorylation system.

MAPK: Mitogen Activated Protein Kinase

MAPKK: Mitogen Activated Protein Kinase Kinase

MAPKKK: Mitogen Activated Protein Kinase Kinase Kinase

In recent years tremendous progress has been made toward the understanding of biological functions of plant MAP kinases with many attempts to identify the function and regulation of MAPK gene families in economically important cereal crops.

Rice can be thought of as being a model plant for all cereals, in particular because the rice genome has been fully sequenced. The first MAPK to be characterised from rice was *OsMAPK12* also known as *BWMK1*. The completed rice genome reveals a total of 17 MAPK genes, and according to the phylogenetic analysis and pairwise comparison of rice MAPKs and the 20 *Arabidopsis* MAPKs, the rice MAPKs can be divided into six groups (A, B, C, D, E and F). The MAPKs belonging to groups A, B and C contain the TEY activation motif, while the other MAPK groups contain TDY activation motif. In more detail, group A consists of two MAPKs (*OsMAP1* and *OsMAPK5*), group B consists of two (*OsMAPK2* and *OsMAPK6*), group C contains two MAPKs (*OsMAPK3* and *OsMAPK4*), group D consists of six MAPKs (*OsMAPK7*, *OsMAPK8*, *OsMAPK9*, *OsMAPK10*, *OsMAPK11* and *OsMAPK12*), group E consists of four MAPKs (*OsMAPK13*, *OsMAPK14*, *OsMAPK15 and OsMAPK16*); finally group F contains one MAPK (*OsMAPK17*) (Liu and Xue, 2007; Rohila and Yang, 2007, Table 1.5, Fig. 1.6).

Many plant MAPKs have been associated with abiotic stresses such as drought and cold (Agrawal *et al.*, 2003a). However, plant MAP kinases have also proved to be involved in defence mechanisms against a wide range of pathogenic fungi (Liu and Kolattukudy, 1997; Hamer and Talbot, 1998; Müller *et al.*, 1999; Takano *et al.*, 2000; Zhang and Gurr, 2001). For example, it was found that expression of *OsMAPK12* was induced 4 hours after challenging the rice leaves with the blast pathogen *M. oryzae*, and also 30 minutes after wounding. Treatment of rice with different compounds such as fungal elicitors, protein phosphatase inhibitor, heavy metals, high salt and sucrose, and the hormones SA, JA and ET were also found to induce the gene expression of *OsMAPK12* as well, suggesting this protein is playing a significant role in response to biotic and abiotic stresses (Agrawal *et al.*, 2003b).

Table 1.5 Rice mitogen-activated protein kinase.

Rice MAPK	Group	T-Loop	Chromosome number
OsMAPK1	A	TEY	6
OsMAPK2	B	TEY	8
OsMAPK3	C	TEY	2
OsMAPK4	C	TEY	6
OsMAPK5	A	TEY	3
OsMAPK6	B	TEY	10
OsMAPK7	D	TDY	5
OsMAPK8	D	TDY	1
OsMAPK9	D	TDY	5
OsMAPK10	D	TDY	1
OsMAPK11	D	TDY	6
OsMAPK12	D	TDY	6
OsMAPK13	E	TDY	2
OsMAPK14	E	TDY	5
OsMAPK15	E	TDY	11
OsMAPK16	E	TDY	1
OsMAPK17	F	TDY	5
From: Reyna and Yang (2006)			

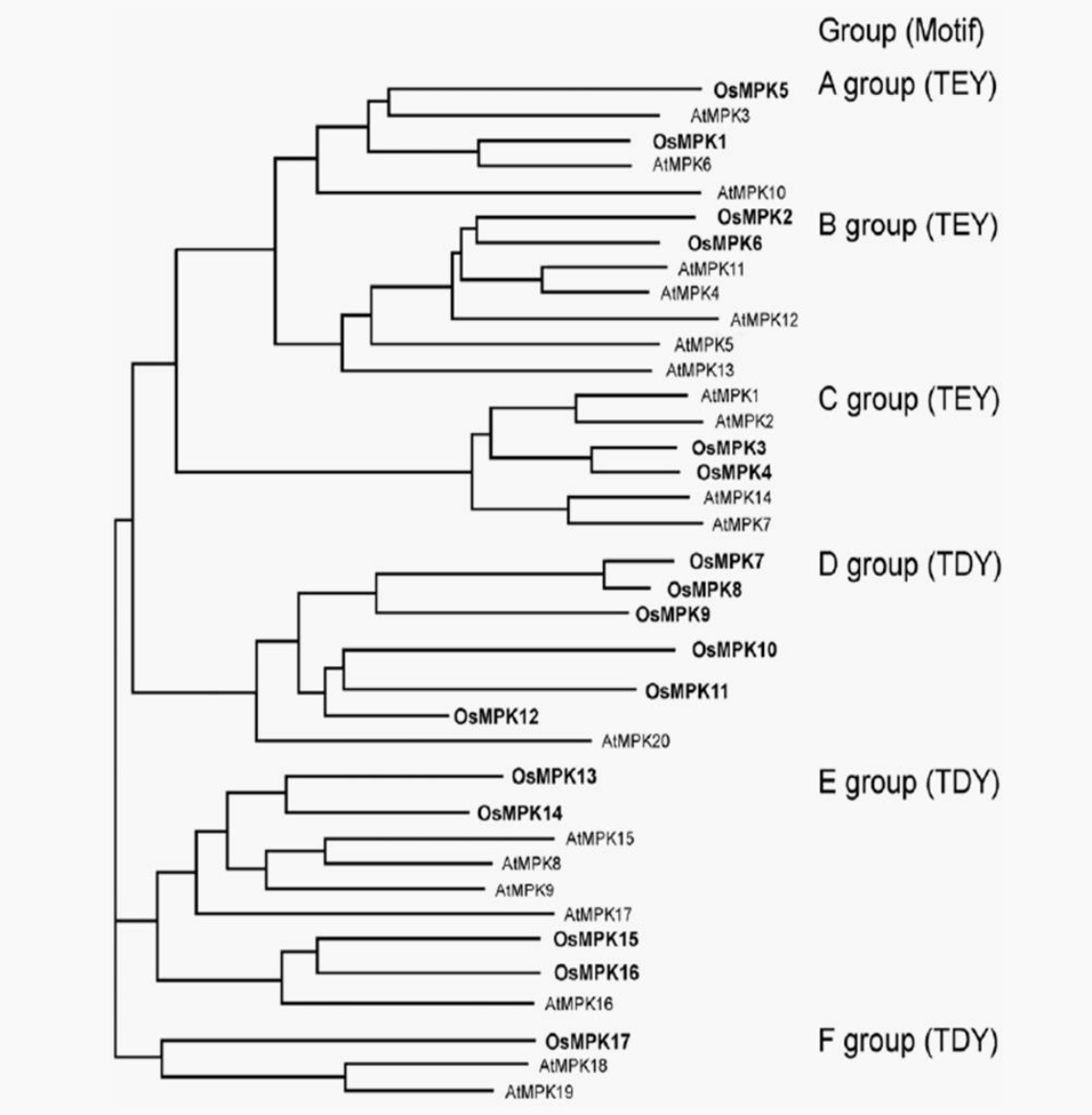


Figure 1.6 Phylogenetic analysis of rice MAPK amino acid sequences in comparison to the 20 *Arabidopsis* MAPKs proteins

From: Reyna and Yang (2006).

There are different approaches for the nomenclature of rice MAPKs by different groups, which leads to confusion, as it is possible to find several different names for one rice MAPK in the literature, for example *OsMAPK1* is also known as *OsMAPK6*, *OsMAPK5* is also known as *OsMAPK2*, *OsMSRMK3* or *OsMAPK4*, *OsMAPK12* is also known as *OsBMPK1*, *OsMAPK13* is also known as *OsBIMK2*. According to Liu and Xue (2007) their proposed naming system covers 16 *OsMAPK* genes, while Hamel *et al.* (2006) cover 15 *OsMAPK* genes. Rohila and Yang (2007) identified 17 rice *OsMAPKs* and they proposed a nomenclature system for new *OsMAPKs* depending on the numerical order in which appears on the phylogenetic tree, and they composed their phylogentic tree according to the phylogentic relationship of the 17 rice *OsMAPKs* in comparison with 20 *Arabidopsis* MAPKs (Table 1.6). In this study the nomenclature system of Rohila and Yang (2007) was followed for naming rice MAPKs.

Table 1.6 Different nomenclature system for rice MAPKs.

Rohila and Yang (20070			Liu and Xue (2007)			Other names
Rice MAPK	Accession ID	Amino acid	Rice MAPK	Accession ID	Amino acid	
OsMPK1	AB183398	398	OsMPK3	AP006533	398	MAPK6, OsSIPK
OsMPK2	AAS79349	394				
OsMPK3	AAG40581	370	OsMPK2	AP005191	370	OsMAPK3
OsMPK4	CAB61889	369	OsMPK5	AP004278	369	OsMAPK2, OsMSRMK3, OsMAPK4
OsMPK5	AF479883	369	OsMPK6	AC134232	369	OsMAPK1, OsMAPK2, OsMSRMK2, OsBMK1
OsMPK6	NM_001071691	376	OsMPK15	AP003725	501	OsMPKG2
OsMPK7	NP_001056342	592	OsMPK4	AC120986	569	
OsMPK8	AJ512643	569	OsMPK1	AP003222	569	WJUMK1, OsMPKG1
OsMPK9	Q6L5D4	700	OsMPK16		369	OsMPK9
OsMPK10	NP_001043642	611	OsMPK8	AP003220	611	
OsMPK11	BAD69155	570	OsMPK13	AP004799	506	OsBIMK2, OsMPK2, OsRMAPK2
OsMPK12	AF177392	580	OsMPK7	AP003621	506	BWMK1
OsMPK13	AY524973	506	OsMPK12	AC098571	581	
OsMPK14	AAS98446	542	OsMPK9	AC104277	542	
OsMPK15	ABA92667	498	OsMPK10	AC135497	498	OsSJMK1
OsMPK16	NM_192298	501	OsMPK14	AP005763	570	
OsMPK17	AAT39148	581	OsMPK11	AC098573	721	

Petersen *et al.* (2000) isolated a mutation in *Arabidopsis thaliana* by a transposon inactivation of *MAPK4*, and this *mpk4* mutant showed observable phenotypes of dwarfism, curled leaves and flowers with reduction in the fertility and pollen production. They attributed the dwarfism to the enhanced SA levels found in *mpk4*, but the *mpk4* mutants respond normally to different environment stresses including desiccation, cold, salt treatment and heat shock, and normal responses were exhibited by *mpk4* mutants to different phytohormones such as auxin, cytokinin, abscisic acid, brassinosteroid and gibberellins.

It was found that *mpk4* mutant plants also exhibited increased resistance to a virulent bacterial pathogen *P. syringae* pv. *tomato* DC3000, and to the oomycete fungal pathogen *Peronospora parasitica* when compared to the wildtype of *Arabidopsis* which showed severe disease symptoms when infected. RNA blots revealed that *mpk4* mutants constitutively expressed different *PR1* genes including *PR1, PR2* and *PR5*, suggesting that *MAPK4* is a negative regulator for SA which drives the expression of *PR* genes and SAR.

The study of Brodersen *et al.* (2006) also revealed that the *Arabidopsis MAPK4* is required for expression of the antifungal *PLANT DEFENSIN 1.2* (*PDF1.2*) gene, which is considered as a marker gene for ethylene/jasmonic acid pathway in resistance to necrotrophic pathogen *Alternaria brassicicola*. Their results showed that *mpk4* mutant plants were more susceptible to the necrotrophic pathogen than the wildtype, and RNA blot results showed there was a reduction in level of *PDF1.2* expression. Also *mpk4* mutant plants failed to respond to JA application when the JA-defence gene expression was checked for example *PDF1.*2, suggesting that *MAPK4* is essential for local resistance to the infection by the necrotrophic pathogen and the *PDF1.2* induction mediated by the ethylene/jasmonic acid defence pathway.

Regarding the downstream target of MAPK4, it was found that the nuclear protein MAP Kinase 4 Substrate 1 (MKS1: which is a 222-amino acid protein) acts as a substrate. Andreasson *et al.* (2005) confirmed the direct interaction between MAPK4 and MKS1 in both yeast two hybrid (Y2H) and *in vitro* assays, and they revealed that this type of interaction is specific, as MKS1 interacted specifically with MAPK4 and did not interact with closely or distantly related MAPKs such as MAPK5 and MAPK17.

Andreasson *et al.* (2005) also generated different *Arabidopsis* lines with modifications to expression of the *MKS1* gene. They overexpressed MKS1 (by using a constitutive promoter CaMV: *35S-MKS1*) and underexpressed *MKS1* (by using RNA interference: RNAi-*MKS1*), the *35S-MKS1* plants exhibited a semidwarf phenotype and showed an increase in the expression of *PR1* at mRNA level, and accumulated more SA (3.8 fold more than the wildtype), and were more resistant to infection by the biotrophic pathogen *P. syringae* pv. *tomato* DC3000, as compared to the RNAi-*MKS1* line which did not exhibited any abnormalities. Their findings suggest that MKS1 substrate is involved in an SA-dependent plant defence pathway.

The downstream targets of MKS1 were found to be the transcription factors *WRKY25* and *WRKY33* by Y2H analysis. The complex of MAPK4-MKS1-WRKY33 was found to exist in the nucleus (Andreasson *et al.*, 2005; Qiu *et al.*, 2008). These transcription factors are involved in repression of the SA mediated defence pathway, the mutants of *Arabidopsis* with overexpression of *WRKY25* and *WRKY33* increased their susceptibility to the biotrophic bacterium *P. syringae*, while the knockout of *WRKY33* (*wrky33* mutant) showed increased susceptibility to the necrotroph fungal pathogens *A. brassicicola* and *Botrytis cinerea* (Zheng *et al*., 2006; Zheng *et al.*, 2007).

The Y2H analysis for protein-protein interaction between *Arabidopsis* MAPKKs (MKKs) and MAPKs showed that nine of the ten MKK proteins interacted with one or more MAPK targets, and revealed that the upstream MKKs for MAPK4 are the closely related MKK1 and MKK2 (Teige *et al.*, 2004, Lee *et al.*, 2008), it was also found that the stress-induced MAPKKK MEKK1 interacts with MKK1 and MKK2 (Qiu *et al.*, 2008).

In rice, a number of published works have focused on the MAPKs and plant disease responses. In the study of Xiong and Yang (2003), they generated rice plants which overexpressed the *OsMAPK5* gene (using the *35S* promoter from the cauliflower mosaic virus) and also developed plants with suppression of expression (using double-stranded RNA interference dsRNAi) of the *OsMAPK5* rice gene. They found that the suppression of *OsMAPK5* gene expression and thus reduction in OsMAPK5 kinase activity resulted in the constitutive expression of *PR1* and *PR10* genes, and these transgenic rice lines showed enhanced resistance to the blast pathogen (caused by *M. oryzae*), while the *OsMAPK5* overexpression rice lines showed a similar susceptibility level to the blast pathogen as in the wildtype without any significant differences in terms of disease severity, lesion number and fungal growth.

The results of Reyna and Yang (2006), when they studied the gene expression profile of rice MAPKs after challenging with blast pathogen (*M. oryzae*) at early (hours) and later (1-3 days) post-inoculation, showed that the blast fungus induces the expression of nine of 17 MAPKs genes *(OsMAPK2, OsMAPK4, OsMAPK5, OsMAPK7, OsMAPK8, OsMAPK12, OsMAPK13, OsMAPK15* and *OsMAPK17*), they also found that four rice MAPKs (*OsMAPK4, OsMAPK5, OsMAPK8* and *OsMAPK13*) were found to be induced in lesion tissue of the Sekiguchi mutant B689A-*sl* (which gives spontaneous reddish-brown lesions).

It was found that *OsMAPK6* plays a pivotal role in rice resistance to the bacterial blight (caused by *Xanthomonas oryzae* pv. *oryzae* strain PX061), the suppression of *OsMAPK6* in rice plants led to enhanced resistance to this pathogen, and the lesion area and bacterial growth decreased significantly compared to the wildtype response. Suppression of this gene in transgenic rice induced the expression of defence-related genes such as *PR5, PR10, PAL1* (phenylalanine ammonia-lyase) and *WRKY30*, and the salicylic acid level were found to be 1.6 fold higher in the *OsMAPK6*-suppresson lines compared to the wildtype; the study suggest that *OsMAPK6* is negatively regulates SAR (Yuan *et al.*, 2007; Shen *et al.*, 2010), it is relevant that *OsMAPK6* is a homologue of *Arabidopsis AtMAPK4,* which suggests a similarity in plant defence mechanisms against biotrophic pathogens.

1.7 The role of phytohormones in plant disease resistance

1.7.1 Salicylic acid

Salicylic acid (or ortho-hydroxy benzoic acid) is a natural phenolic compound present at different levels in a wide range of plant species (Raskin *et al.*, 1990). While the medical importance of SA and its acetylated derivative, aspirin, has been widely known for a long time, the importance of SA for plant life has just emerged in the last decades (Dempsey *et al.*, 1999)

Different biological studies have suggested that SA is synthesized from phenylalanine (Fig. 1.7). SA has been proven to play a pivotal role in plant signal transduction systems as a major component in the development of the systemic acquired resistance (SAR) response (Antoniw and White, 1982). The first mention that SA is involved in the regulation of plant resistance was by White (1979), when he noticed the induction of disease resistance of tobacco plants to tobacco mosaic virus after the exogenous application of SA. In addition, the systemic increases in SA in both tobacco and cucumber plants during the infection with TMV and *Pseudomonas syringae* was the first evidence for the role of SA as a resistance signal (Malamy *et al.*, 1990; Metraux *et al.*, 1990; Rasmussen *et al.*, 1991).

It was found that transgenic tobacco and *Arabidopsis thaliana* expressing the bacterial salicylate hydroxylase (*nahG*) gene, which encodes an enzyme that converts SA to catechol, failed to accumulate SA; these transgenic plants were unable to induce SAR, showed a reduction or no *PR* gene expression after infection and showed high susceptibility to different pathogens (Delaney *et al.*, 1994).

SA levels were found to have increased as a response to pathogen attacks in different plants such as tobacco, *Arabidopsis* and cucumber, the increase in SA level was accompanied by SAR (Malamy *et al.*, 1990; Uknes *et al.*, 1992; Dempsey and Klessig, 1995). *OsPR1a* and *OsPR1b* mRNA was found to be up-regulated in the rice seedlings 24h and 48h after treatment with exogenous SA at a concentration of 100 µM (Agrawal *et al.*, 2001).

In the earlier studies on the interaction between SA and SAR, SA was considered as a translocated signal for SAR. Metraux *et al.* (1990) reported an increase in the level of SA in the phloem of cucumber plants after inoculation with tobacco necrosis virus or the fungus *Colletotrichum lagenarium*, and this increase was followed by the development of SAR. In tobacco plant, Shulaev *et al.* (1995) labelled SA with oxygen-18 ($^{18}O_2$) by depending on the fact that SA biosynthesis in tobacco plant is by the O_2-dependent hydroxylation of benzoic acid, hence, they enclosed tobacco plants in an $^{18}O_2$ rich environment, after inoculation of tobacco plant with TMV, they found that the 70% of SA in the upper uninoculated leaves was labelled with $^{18}O_2$ and had been produced and transported from the infected tobacco leaf. In addition, they found an induction of expression of PR1 protein in these upper uninoculated leaves.

Regarding the antimicrobial properties of SA, a lot of attention has been given to clarify this activity; Qin *et al*. (2003) have reported that an SA concentration of 100 mM was toxic to the spore germination and germ tube elongation of two fungal pathogens, *Penicillium expansum* and *Alternaria alternata*. Similar antifungal activity was found with different plant pathogen such as *Fusarium oxysporum* f.sp. *niveum,* where it was found that an SA concentration of 5.8 mM was sufficient to inhibit the *in vitro* growth of mycelia of this pathogen. In addition, a severe repression of conidial germination and sporulation were reported at all SA concentrations (0.7-5.8 mM) (Wu *et al.*, 2008).

In relation to the importance of SA for plant life, it was reported that the exogenous application of SA and related compounds such as benzothiadiazole (BTH) enhanced the growth parameters for different group of plants. Khan *et al.* (2003) have reported that the foliar treatment of SA at concentrations of 1 mM and 10 µM enhanced the photosynthesis rates, stomatal conductance and transpiration, as well as leaf area and plant dry mass was increased in both corn and soybean plants. Similar growth promotion was reported in barley and wheat plants after grain treatment with SA at concentrations of 100 µM, this application led to an increase in germination, leaf number, fresh and dry weight per seedling (Pancheva, 1996; Hayat *et al.*, 2005). Apart from the growth promoting effect of SA, it was also reported that exogenous application of SA effectively alleviated the inhibitory effect of abiotic stresses such as the toxic effect of heavy metals on many plants species.

In barley seedlings the application of exogenous SA as a seed soaking treatment (0.5 mM) was found to be enough to alleviate the toxic effect of cadmium (Metwally *et al.* 2003). Similar beneficial effects were reported for lead and mercury tolerance in rice plant, the application of SA decreased the deterioration of the membranes in the leaves of rice which is caused by an increase in lipoxygenase activity after treatment with 10 µM $PbCl_2$ or 10 µM $HgCl_2$ (Mishra and Choudhuri, 1999; Zhou *et al.*, 2009). Salicylic acid plays a significant role in providing tolerance to the plants when exposed to another set of abiotic stresses such as water stress (drought or flooding), high and low temperatures and salinity (Shakirova *et al.*, 2003; Taşgín *et al.*, 2003).

Regarding *NPR1* gene (Non-expresser PR1) which considered as a regulatory gene of systemic acquired resistance pathways and plays an essential role in signalling pathways. *NPR1* was cloned first time by Cao *et al.* (1997) in *Arabidopsis* plants; their results showed that the *Arabidopsis NPR1* gene encodes a protein with ankyrin repeats which is important for protein-protein interaction, and they found that the disruption of the ankyrin repeat consensus sequence by conversion of a cysteine to a tyrosine of NPR1 protein in *Arabidopsis* mutant *npr1* exhibited a low level of *PR1* gene expression after treatment with salicylic acid compared to the wildtype. Suggesting that the ankyrin repeats are important for *NPR1* function.

Several elegant studies were focused on the molecular function of *NPR1* gene; the results of Cao *et al.* (1998) showed that the overexpression of *NPR1* gene in *Arabidopsis* plants confers resistance to different pathogens including *Pseudomonas syringae* and *Hyaloperonospora parasitica*. This result suggests that the *NPR1* gene is a positive regulator for systemic acquired resistance.

The mechanism by which *NPR1* gene involved in systemic acquired resistance and their responsive gene expression has been studied by several scientist groups. Kinkema *et al.* (2000) succeed to fuse the *NPR1* cDNA with green fluorescent protein (GFP) to localize the *NPR1*in the plant cells. The NPR1-GFP fusion protein was found to be accumulated in the nucleus as a response to several SAR inducers. The nuclear localization of *NPR1* has an important role in for regulation of *PR1* gene expression. The study of Mou *et al.* (2003) proved that the NPR1 protein is present in two forms, uninduced oligomeric NPR1 protein which form through intermolecular disulphide bonds and present in cytoplasm; the other form is induced monmeric NPR1 protein which accumulated in the nucleus and activates gene expression.

The mechanism by which the oligomer NPR1 releases the active form of NPR1 (monomer NPR1) is explained by the role of redox status in the plant cell (Spoel and Loake, 2011), its well known that the elevated level of salicylic acid induces the change in redox status which resulted in disulphide reduction and *S*-nitrosylation of specific cysteine in NPR1 protein. This reduction in disulphide releases NPR1 monomer and then translocated into their target in nucleus to induce *PR1*gene expression (Mou *et al.*, 2004; Spoel *et al.*, 2009). NPR1 protein at the nucleus is thought to interact with TGA transcription factor conserved basic leucine zipper protein, and the complex of NPR1-TGA transcription factor activates the expression of defensive genes (Johnson *et al.*, 2008; Tada *et al.*, 2008). Finally, the NPR1 active monomer form is also involved in suppression of jasmonic acid signalling in the plant cells cytosol (Spoel *et al.*, 2003).

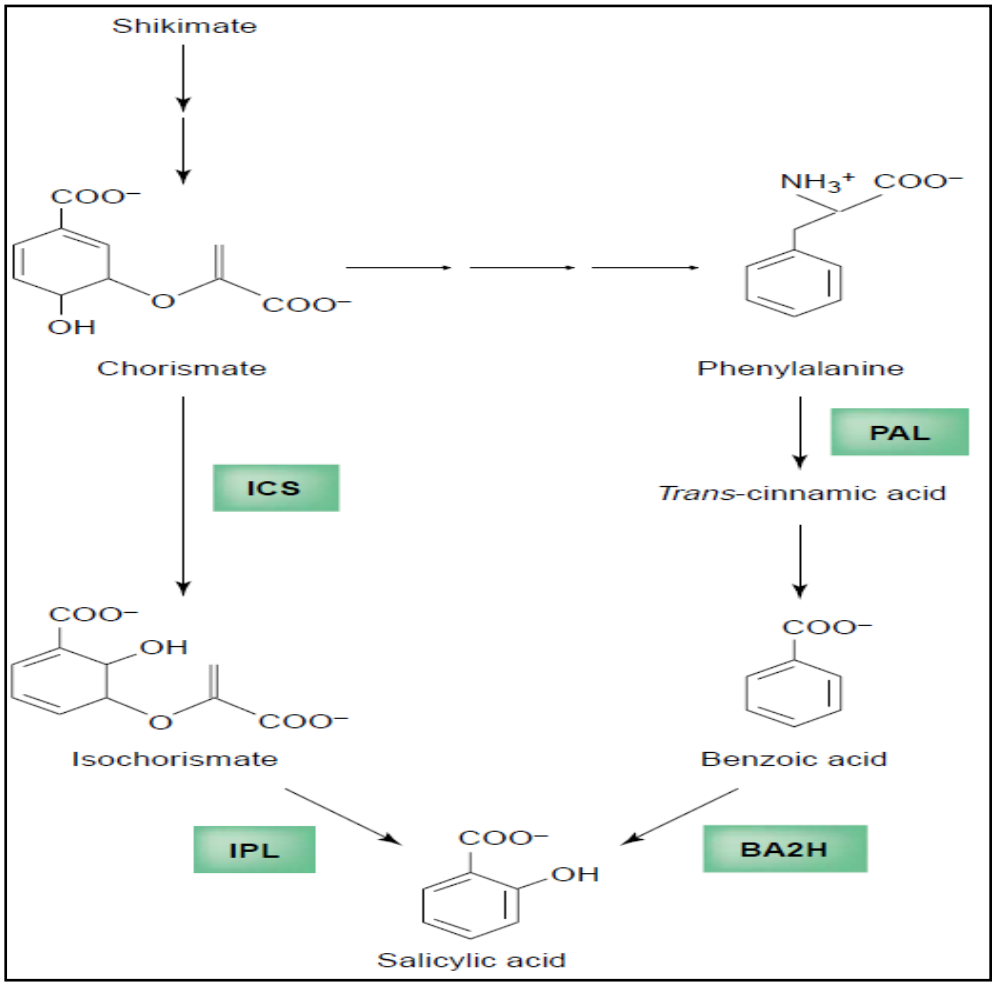


Figure 1.7 Proposed pathways for the biosynthesis of SA in plants.

PAL: phenylalanine ammonia lyase, BA2H: benzoic acid-2-hydroxylase, ICS: isochorismate synthase, IPL: isochorismate pyruvate lyase.

From: Shah (2003).

1.7.2 Jasmonic acid

Jasmonic acid (JA) and its methyl ester (methyl jasmonate MeJA) are a family of cyclopentanone derivatives with regulatory importance in the life of the plants including growth, developmental processes such as seed germination, root growth, ripening and fertility, as well as defence and wound responses (Creelman and Mullet, 1997; Wasternack and Parthier, 1997).

The biosynthesis of jasmonates mainly depends on the peroxidation of fatty acids in plants, more specifically on the synthesis of linolenic acid via the octadecanoic pathways which is catalyzed by lipoxygenase (LOX), allene oxide synthase (AOS), allene oxidecyclase (AOC), and 12-oxo-phytodienoic acid reductase (OPR), and followed by three cycles of β-oxidation (Fig. 1.8). The first identification of MeJA was attained in several plant species as a component of essential oils, and it was first isolated from extracts of the jasmine plant (*Jasminum grandiflorum*) in 1962 (Creelman and Mullet, 1997; Ellis and Turner, 2001) and it has since been identified in over 160 diverse plant families (Sembdner and Parthier, 1993).

It was found that the application of MeJA at concentration of 0.1µM inhibited 50% of the primary root growth of wildtype of *Arabidopsis thaliana* (Staswick *et al.*, 1992). Another biological functions of JA in plants is its role in pollen maturation and flower opening; in the study of Ishiguro *et al.* (2001) on an *Arabidopsis* mutant defective in anther dehiscence1 (*dad1*), it was found that JA accumulation was reduced significantly in the *dad1* flower buds, but the exogenous application of JA recovered the defects in this mutant. It has been found that the levels of JA increased in different plants as a response to drought and water stress; the spears tips of *Asparagus officinalis* when exposed to drought showed an increase in the level of MeJA, and exposing *Pinus pinaster* to extreme water stress induced these plants to accumulate high levels of MeJA (Gapper *et al.*, 2002; Pedranzani *et al.*, 2007). More recently, Jubany-Marí *et al*. (2010) found that the Mediterranean shrub (*Cistus albidus* L.) when exposed to drought showed enhanced levels of MeJA after 11 weeks of treatment and reached 400 pMol/g of dry weight.

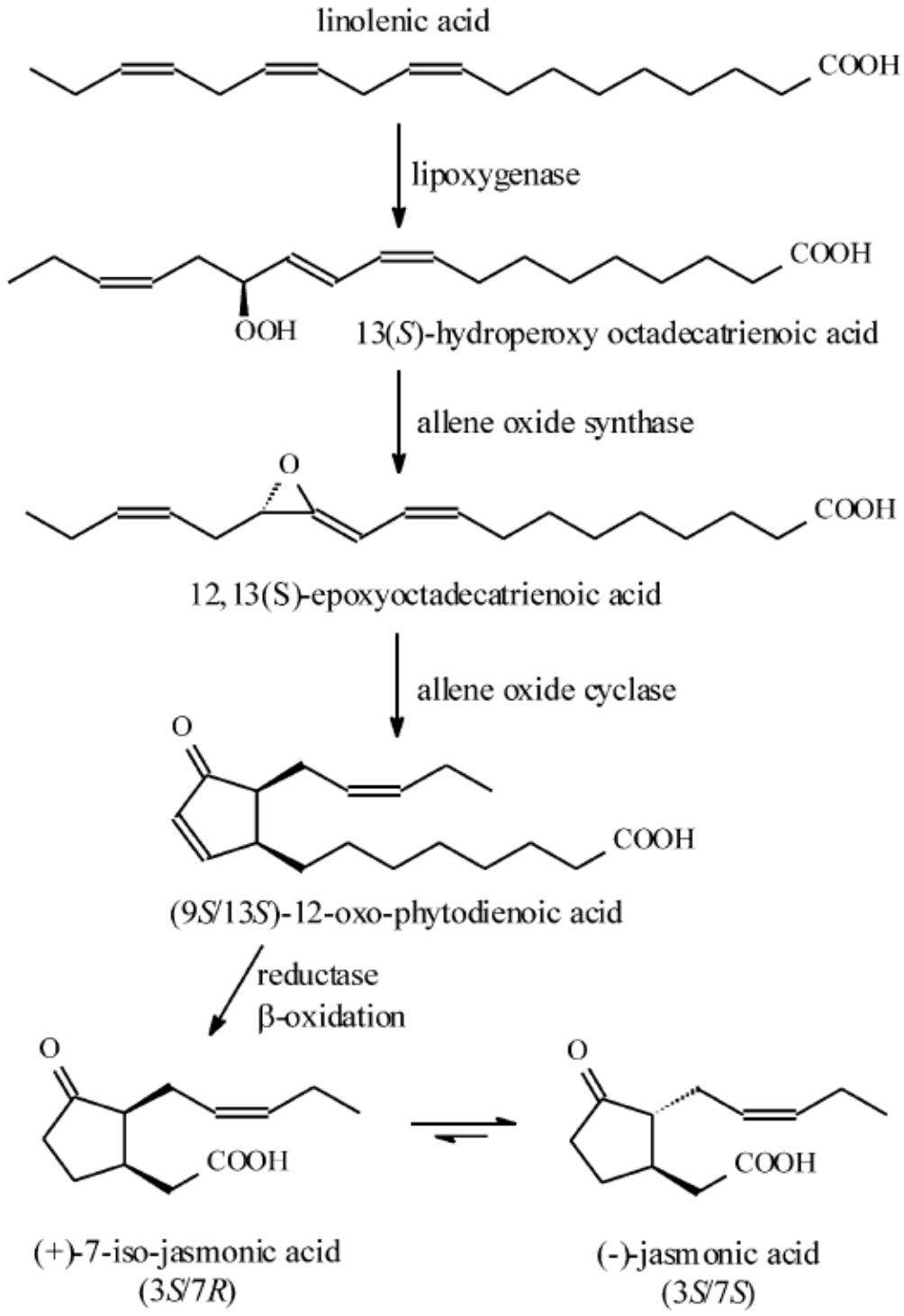


Figure 1.8 Biosynthetic pathway of JA.

From: Ziegler *et al.* (2001).

The role of JA in plant disease resistance is mainly dependent on the type of the pathogens; Thomma *et al.* (1998) reported that the *Arabidopsis* mutant *coi1*, which negatively affects the perception of JA, shows enhanced disease susceptibility to infection by necrotrophic pathogens such as *A. brassicicola and B. cinerea*, but not to the biotrophic pathogen *Hyaloperonospora parasitica* (previously known: *Peronospora parasitica*). In wildtype *Arabidopsis*, they revealed the up-regulation of different responsive genes such as *PDF1.2* (the antimicrobial protein defensin), *PR3* and *PR4* in response to the attack with the necrotrophic pathogen *A. brassicicola*. However, Schweizer *et al.* (1997) found that the infection of rice leaves with the hemibiotrophic pathogen *M. oryzae* did not enhance the level of endogenous JA compared with the non-treated control, while JA application induced the accumulation of several pathogenesis-related proteins, such as *PR1, PR3, PR5* and *PR9*, but this exogenous application did not confer any resistance against the rice blast pathogen.

The treatment of wheat leaves with MeJA (300 µM) induced at least eight defence genes including *PR1.1, PR2, PR3, PR4, PR5, PR10, TaPERO* (peroxidise) and *TaGLP2* (germin-like), the treatment with MeJA before inoculation of wheat plants with the crown rot necrotrophic pathogen (*Fusarium pseudograminearum*) delayed the development of necrotic symptoms for two weeks (Desmond *et al.*, 2005). Treating *Medicago truncatula* plants with MeJA induced partial resistance against the charcoal rot pathogen (*Macrophomina phaseolina*), it was found that treated Medicago plants (4 week old with 0.1% v/v MeJA) developed disease symptoms more slowly and survived longer than the control plant which died after four days of infection, while treated plants still survive up to 7 days (Gaige *et al.*, 2010).

Enhanced disease resistance was shown in transgenic *Arabidopsis* plants overexpressing the *JMT* gene which encoding an S-adenosyl-l-methionine: jasmonic acid carboxyl methyltransferase (which is responsible for catalyzing the JA methylation to form methyl jasmonate), these transgenic plants showed enhanced levels of resistance toward the necrotroph fungal pathogen *B. cinerea* and exhibited a constitutive expression of jasmonate- responsive genes such as *VSP* (vegetative storage protein) and *PDF1.2* (Seo *et al.*, 2001). The *VSP* and *PAD4* genes are used as marker genes in *Arabidopsi*s and other plants for characterization of the jasmonate-dependent defence responses (Brown *et al.*, 2003; Brodersen *et al.*, 2006).

The jasmonate inducible gene *JIOsPR10 (*jasmonate inducible rice pathogenesis-related protein class 10) was found to be up-regulated in rice plants after the treatment of rice seedling leaves (two weeks old) with JA at a concentration of 100 µM, the expression profile of this gene showed a strong increase in expression after 48 and 72 hours of treatment, the results showed that this gene might be involved in defence or stress responses (Jwa *et al.*, 2001).

In addition, expression of different defence genes was observed when JA was applied exogenously, e.g. the treatment of cell suspension cultures of *Vitis vinifera* with 20 µM of MeJA stimulated the expression of phenylalanine ammonia lyase, chalcone synthase, stilbene synthase, UDPglucose:flavonoid-O-glucosyltransferase, proteinase inhibitor and chitinase genes (Belhadj *et al*., 2008).

Furthermore, the application of MeJA can provide different plants with systemic protection against plant pathogens. Meir *et al.* (1998) reported that the treatment of rose (*Rosa hybrid*) petals with different concentrations of MeJA (50-300 µM) led to inhibition of the decay disease caused by the grey mould pathogen *B. cinerea*. The concentration of 300 µM MeJA prevented the pathogen from establishing the disease on rose plants, and the results showed a direct antifungal activity of MeJA on the growth of pathogen *in vitro*, the spore germination and germ tube elongation were inhibited at 400 µM of MeJA. In addition the application of JA can activate separate sets of biosynthetic genes which lead to the accumulation of antimicrobial secondary metabolites, such as alkaloids, terpenoides, flavonoids, anthraquinones and glucosinolates (Memelink *et al.*, 2001).

In peach fruit (*Prunus persica* cv. Bayuecui) it was found that the exogenous application of MeJA (200 µM) induced the resistance of the fruit to brown rot and blue mould diseases caused by *Monilinia fructicola* and *P. expansum*, respectively, as well as a direct inhibition by MeJA on *P. expansum* growth i*n vitro* (Yao and Tian, 2005). More recently, the induced resistance of tomato fruit to infection with the necrotrophic pathogen *B. cinerea* was achieved by treatment with MeJA (100 µM), this treatment led to reductions in the disease symptoms (lesion size) in tomato fruit of up to 42.5% (Yu *et al.*, 2009).

The importance of JA as a defence hormone against arthropod herbivores came from the discovery that this hormone is a strong elicitor of proteinase inhibitor (PI) expression in tomato (Farmer and Ryan, 1990). Farmer *et al.*, (1992) reported that the exposure of young tomato leaves to methyl jasmonate vapour induced the accumulation of two protein inhibitors I and II, they found similar results with alfalfa plants which produced alfalfa trypsin inhibitors when they exposed these plants to MeJA vapour, and these protein inhibitors are considered as anti-feedants for different harmful insects. More recently, it was found that foliar application of JA at concentrations of 2.5-5 mM induced systemic resistance in rice and reduced the survival of rice brown hopper (*Nilaparvata lugens*) (Senthil-Nathan *et al.*, 2009). In addition to rice, JA and its derivatives have been proved to induce resistance in different hosts such as potato plant to late blight pathogen (*Phytophthora infestans*), celery to leafminers, wheat plant to the fungal pathogen *Stagonspora nodorum* (Il'inskaya *et al.*, 2000; Black *et al.*, 2003; Jayaraj *et al.*, 2004).

To summarize the role of jasmonic acid and their responsive genes in plant disease resistance, it was found that the exogenous application of jasmonic acid leads to accumulate different sets of jasmonic acid responsive genes. Penninckx *et al.* (1998) found that the transcript levels of antimicrobial defensin protein PDF1.2 was up-regulated significantly as a response to jasmonic acid application, similar increase was reported in *Arabidopsis* plants with the antimicrobial protein thionin (THI2.1) (Epple *et al.*, 1995). Memelink *et al.* (2001) showed that the accumulations of different antimicrobial secondary metabolites were increased after jasmonic acid application such as alkaloids, terpenoides, flavonoides, anthraquinones and glucosinolates.

An elegant micro-array study of Schenk *et al.* (2000) showed that the exogenous application of methyl jasmonate leads to substantial changes in different *Arabidopsis* gene expressions which involved in oxidative burst and apoptosis, including catalase, glutathione *S*-transferase (GST1) and cysteine protease. Additionally, different defence response genes (antimicrobial genes) were up-regulated as a response to methyl jasmonate application such as *PRs, PDF1.2, PAL* genes and thaumatine-like proteins.

Beside the defence response genes, it was found that the exogenous application of jasmonic acid leads to accumulate high level of *VSP1* gene in *Arabidopsis* which encodes a storage protein in vegetative organs (Guerineau *et al.*, 2003).

1.7.3 Ethylene

Ethylene is an important plant hormone, which plays a crucial role in normal plant development, physiology and response to stresses. Ethylene is essential for many physiological processes such as seed germination, tissue differentiation, the formation of root and shoot primordia, root elongation, lateral bud development, flower initiation, anthocyanin synthesis, flower opening and pollination, ripening, the production of volatile compounds in fruits, leaf and fruit abscission as well in plant responses to biotic and abiotic stresses (Deikman, 1997; Lelièvre *et al.*,1997; Grichko and Glick, 2001).

Ethylene is produced in different parts of plant tissues. *S*-adenosylmethionine (SAM) is the precursor of ethylene biosynthesis which is produced by conversion of methionine by SAM synthase, according to the Yang cycle the first step of ethylene production is the conversion of SAM to ACC (aminocyclopropane-1-carboxylic acid) by ACC synthase (*S*-adenosylmethionine methylthioadenosine-lyase) and the other product of this reaction is 5-methylthioadenosine (MTA) which is converted to methionine. The final step in ethylene biosynthesis is the formation of ethylene by oxidizing the ACC by ACC oxidase, in addition to ethylene, CO_2 and cyanide are also produced, and cyanide is a highly toxic compound which is detoxified by β-cyanoalanine synthase to form the nontoxic compound β-cyanoalanine (Wang *et al.*, 2002; Stearns and Glick, 2003, Fig. 1.9).

The perception of ethylene in plants was first identified in *Arabidopsis*, there are five identified ethylene proteins which are membrane-localized receptors: ethylene receptor1, 2 (ETR1, ETR2), ethylene insensitive 4 (EIN4), ethylene response sensor 1 and 2 (ERS1 and ERS 2) (Stepanova and Ecker, 2000), all of these proteins bind ethylene with help of a copper cofactor in the transmembrane domain, and three of these five receptor proteins (ETR1, ETR2 and EIN4) have receiver domains which are thought to donate a phosphate to the CTR1 protein (CONSTITUTIVE TRIPLE RESPONSE 1), this protein is required for the activation of EIN2, which is a cytoplasmic protein that is thought to be an activator for the EIN3 protein with DNA binding activity.

The target of activated EIN3 is thought to be ethylene response factor genes (ERF1), as well as EIN3-like proteins (EIL1 and EIL2) which are thought to act as transcription factors, ERF1 belongs to a large family of plant transcription factor known as EREBPs (Ethylene-Response-Element-Binding-Proteins), these proteins are recognized by their

ability to bind the DNA motif GCC-box which is associated with ethylene and pathogen-induced gene expression (Kieber *et al.*, 1993; Solano *et al.*, 1998; Stepanova and Ecker, 2000; Wu *et al.*, 2002, Fig. 1.10).

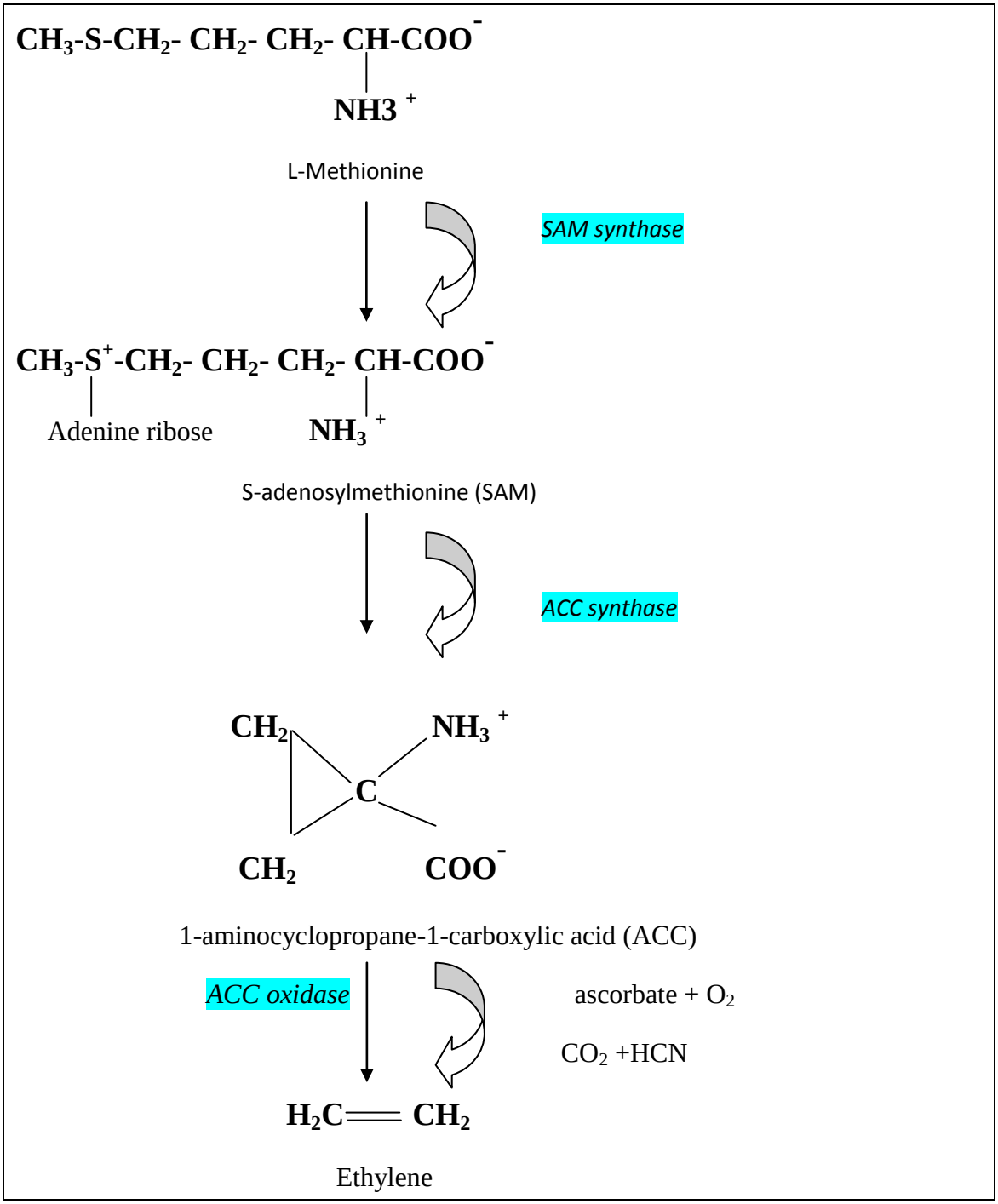


Figure 1.9 Ethylene biosynthesis by ACC synthase and ACC oxidase (ACCO). From: Bassan *et al.* (2006).

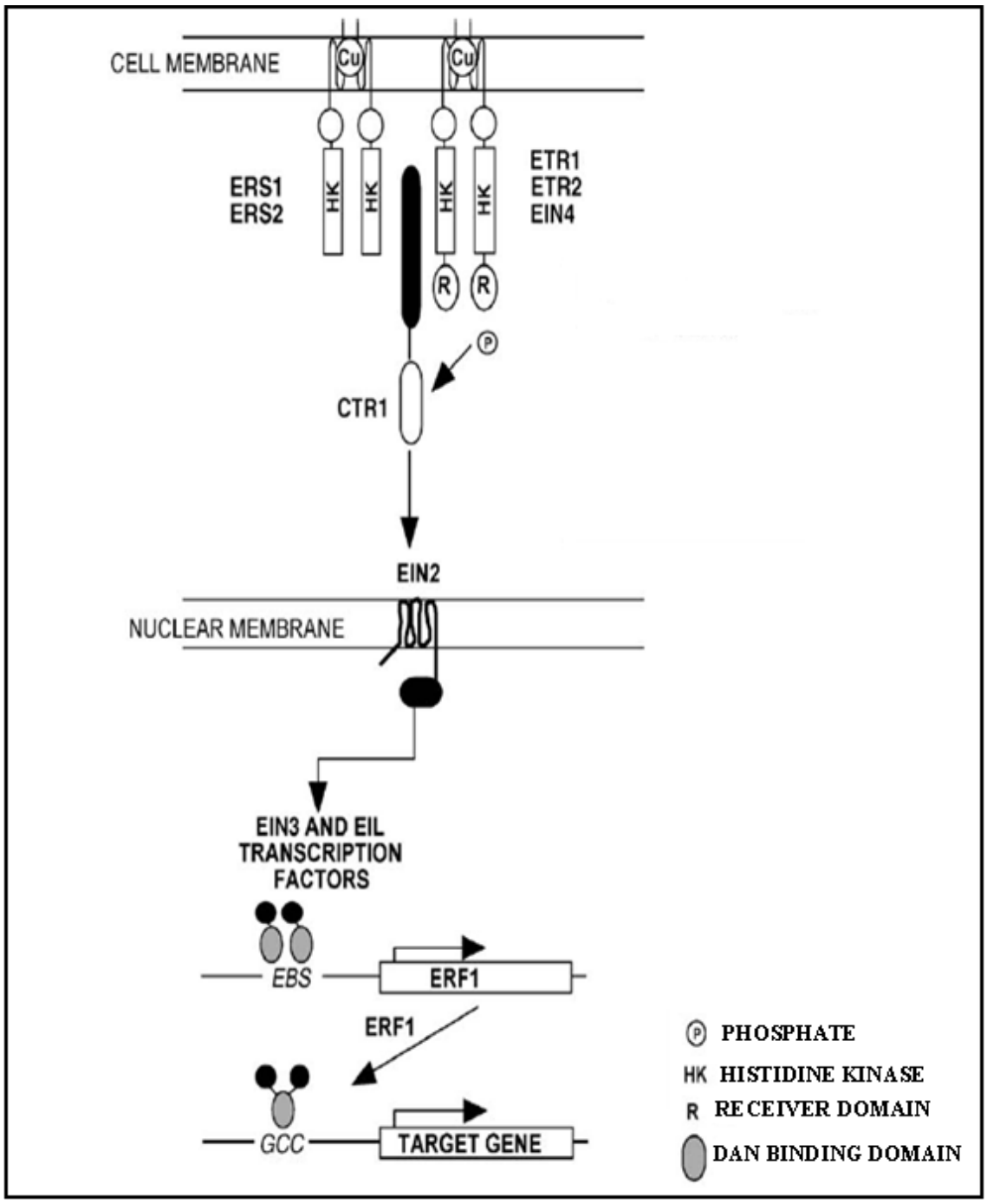


Figure 1.10 Ethylene signal transduction pathway in the plant.

CTR1: constitutive triple response, **EBS**: ethylene binding sequence, **EIL**, **EIN3**- like proteins, **EIN2**, EIN3, **EIN4**: ethylene insensitive, **ERF1**: ethylene response factor, ERS1, 2: ethylene response sensor, **ETR1**, 2: ethylene receptor, **GCC**: GCC box.

From: Stepanova and Ecker (2000).

Depending on the lifestyle of the plant pathogen and the plant species, the role of ethylene in disease resistance pathways is found to differ (Diaz *et al.*, 2002), Hoffman *et al.* (1999) found that the mutants of soybean plant (*Glycin max*) with reduced sensitivity to ethylene respond in different ways to different pathogens, these mutant lines developed less disease symptoms (chlorosis) when challenged with bacterial and fungal pathogens *P. syringae* and *Phytophthora sojae*, respectively, whereas these ethylene-insensitive lines of soybean exhibited more susceptibility to other fungal pathogens including *Septoria glycines* and *Rhizoctonia solani*. It was also found that challenging the *Arabidopsis* ethylene-insensitive *ein2* mutant with necrotrophic pathogen *B. cinerea* showed more sensitivity to the pathogen compared to wildtype plants (Thomma *et al.*, 1999). All of these results suggest that the ethylene has a negative effect on necrotrophic pathogens and leads to a decrease in severity of the disease.

On the other hand, the exogenous application of ethylene can cause an increase in the disease severity in different host-pathogen interactions, the disease severity of the pathogen *Colletotrichum lagenarium* on cucumber plants was increased after treatment with ethylene compared with non-treated cucumber, and similar results were revealed by exogenous application of ethylene to different lines of wheat plant which were found to be more sensitive to the infection by pathogen *Septoria nodorum,* while the application of ethylene synthesis inhibitor (AVG: aminoethoxyvinylglycine) led to a decrease in the severity of disease in cotton plants with the fungi *Alternaria macrospora* and *A. alternata* (Biles *et al.*, 1990; Hyodo, 1991; Bashan, 1994). Ethylene was found to stimulate different plant defence genes expression such as *PR1b1* and *PR1a2* in tomato plants (Tornero *et al.*, 1997). The treatment of carrot plants with ethylene led to accumulation of the phytoalexin 6-methoxymellin (6-ME) in carrot roots, suggesting that phytoalexin production requires ethylene action (Fan *et al.*, 2000).

The *ERF* transcription factor has a pivotal role in the regulation of the *PR* gene expression as it binds the DNA motif GCC-box (Chakravarthy *et al.*, 2003). In *Arabidopsis* plants, transgenic plants overexpressing the *ERF* gene showed enhanced resistance to the fungus *B. cinerea* (Berrocal-Lobo *et al.*, 2002). It was shown that the rice transcription factor *OsBIERF3* when expressed in tobacco confers plants with a

high resistance level toward tomato mosaic virus and wild fire bacterial pathogen *P. syringae* pv. *tomato,* and the expression of the *PR-1* gene was found to be elevated in these transgenic lines when compared to wildtype. Salt tolerance (400 mM NaCl) was also high in transgenic tobacco-*OsBIERF3,* seed germination and growth performances were significantly higher than the wildtype when treated with NaCl (Cao *et al.,* 2005). Similar results were found in the study of Qin *et al.* (2006), transgenic tobacco plants overexpressing *GbERF* showed an increase in the expression profile of different ethylene-inducible genes including *PR2, PR3* and *PR4* compared with WT, also these transgenic lines were more resistant to the bacterial pathogen *P. syringae* pv. *tabaci* in contrast with WT plants

1.8 Reactive oxygen species

Reactive oxygen species (ROS) is a collective term that refers to radicals and non-radicals, derived from the oxygen molecule (O_2). There are different kinds of ROS in plants such as hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-), hydroxyl radical (OH), singlet oxygen ($^1O_2$) and nitric oxide (NO) (Lamb and Dixon, 1997). A lot of attention has been given to H_2O_2 and its role in plant life under different conditions. H_2O_2 has been proven to play a significant role in many physiological processes such as reinforcement of the cell wall, production of phytoalexin, photorespiration and photosynthesis, cell cycle and stomatal movement (Dempsey and Klessig, 1995; Noctor and Foyer, 1998; Mittler *et al.*, 2004, Bright *et al.*, 2006). H_2O_2 at low concentrations is important as a signal molecule in triggering tolerance to biotic and abiotic stresses, and at high concentration H_2O_2 is involved in programmed cell death (Dat *et al.*, 2000), H_2O_2-induced cell death is not only important in the plant response to the biotrophic pathogen attack, but also essential for different developmental processes such as aleurone cell death and allelopathic plant-plant interaction (Apel and Hirt, 2004).

ROS production in the plant as a response to pathogen attack is considered as one of the earliest events in plant defence (Bolwell and Wojtaszek, 1997). The accumulation of ROS in plant defence is biphasic, starting with a rapid but weak transient, then followed by a stronger and prolonged accumulation (Grant and Loake, 2000), thus, different mechanisms have been proposed to explain the ROS generation in plant; the NADPH-dependent oxidase system which is linked with $O2^-$ production in response to pathogen attack, the second system of ROS generation involves germin-like oxalate oxidase

which generates H_2O_2 from oxygen and oxalic acid, the third one is amine oxidase which release H_2O_2 by oxidizing various form of amines (Sagi and Fluhr, 2001; Hu *et al.*, 2003; Walters, 2003).

H_2O_2 is a species of ROS which plays an essential role in plant defence, either by the direct toxic effect on the growth of pathogens, or through its role in induction of different set of defensive genes such as *PR1* and phytoalexin, resistance genes (*R*) and induction of hypersensitive reaction (HR), as well (Peng, 1992; Hammond-Kosack and Jones, 1996).

Lin *et al.* (2005) have reported that the treatment of rice cell suspensions with the PAMP chitosan induced the production of H_2O_2 and increased the activities of phenylalanine ammonia lyase (PAL) and chitinase, in addition, they found that the transcription of defence-related genes such as β-1,3-glucanase (*glu),* chitinase (*chi*) and *PR1* were increased in these cells, while the treatment of rice cells with chitosan and different scavengers (which detoxify H_2O_2) such as catalase and ascorbate blocked the defence response against the blast pathogen (*M. oryzae*).

The results of Grant *et al.* (2000) showed that the northern blot analysis revealed a high transcriptional level of glutathione *S*-transferase (*Gst1*) in *Arabidopsis* up to 18 fold post-inoculation with *P. syringae* pv. *tomato* strain DC3000. This gene is used as a molecular marker for ROS accumulation. Furthermore, they used different variety of protein kinase inhibitors to test their ability to block H_2O_2-induced *gst1* gene expression; they found that these inhibitors were able to inhibit the expression of *gst1*, suggesting a possible role of MAP kinase cascades in *gst1* gene expression in response to reactive oxygen species production. Additionally, their results of in-gel kinase assay (by using myelin basic protein MBP as a substrate) showed that the infiltration of *Arabidopsis* leaf tissues with glucose/glucose oxidase leads to induce two MBP kinases (about 48 and 46-KDa) from a low basal level of activity, suggesting that these two *Arabidopsis* MBP kinases belong to stress-induced class of MAP kinase.

The direct antimicrobial activity of H_2O_2 has been reported for different plant pathogens and the actual toxicity of H_2O_2 is mainly dependent on the sensitivity of the pathogen (Walters, 2003; Shetty *et al.*, 2007). For example a concentration of 0.1 mM of H_2O_2 was sufficient to inhibit the bacterial growth of *Pectobacterium carotovora* (previously known as *Erwinia carotovora*) completely, and 95% of the growth of the pathogen *P. infestan*s was inhibited at similar concentration of H_2O_2, while the growth of

hemibiotrophic pathogen *Septoria tritici* was inhibited significantly at H_2O_2 concentration of 5 mM (Wu *et al.*, 1995; Shetty *et al.*, 2007).

Because of the highly toxic effect of ROS on the plant cells, the production and accumulation of ROS in the plant is under tight control, this control includes oxygen radical detoxifying enzymes, known as scavenging enzymes. These play a crucial role in antioxidant defence mechanism, such as catalase (CAT), superoxide dismutase (SOD), peroxidase (POD). In addition antioxidant molecules such as reduced glutathione and ascorbic acid aid the plant cell to control ROS levels (Karthikeyan *et al.*, 2007). Catalase, which converts H_2O_2 into H_2O and O_2, is considered the most abundant scavenger enzyme in photosynthetic cells (Asada, 1999).

1.9 Aims of the study

The main aim of this study was focused on the biological function of the barley MAP kinase 4 (*HvMAPK4*), more specifically the role of this MAP kinase in plant-pathogen interaction, and to achieve this aim, different transgenic barley were produced with modification in their expression of MAP kinase gene. This particular gene was chosen for analysis because studies on rice have shown that this gene is upregulated in response to infection of rice by the fungal pathogen *Magnaporthe oryzae* (Reyna and Yang, 2007). One form of the transgenic plants was designed to overexpress the *HvMPK4* gene, thus increasing the level of MAP kinase 4 protein, while the other form was antisense for *HvMPK4*, with reductions in the level of MAP kinase 4 protein.

Immature embryos of barley cultivar Golden Promise were used to produce the transgenic plants via *A. tumefaciens*-mediated transformation with the hygromycin resistance gene (hygromycin phosphotranferase: *hpt*) as a selectable marker.

Once the transgenic plants were produced, challenges with the hemibiotrophic fungal pathogen *M. oryzae* was carried out, together with the wildtype and control transgenic barley (transformed with the empty binary vector pBWVec.8), and the response of these plants to the pathogen was investigated during the time course of infection in terms of disease symptoms, endogenous phytohormone content including SA, JA and ET, reactive oxygen species (H_2O_2), *PR-1* gene expression profile (as a marker gene for SAR) and catalase activity.

CHAPTER 2

MATERIALS AND METHODS

2.1 Barley cultivar and growth conditions

The barley cultivar used in this work was *Hordeum vulgare* L. Golden Promise (a spring cultivar), the growth conditions for the donor plants were 18 °C under 16 h photoperiods (light level of 450 µE $.m^{-2}.s^{-1}$ at head light) and 60-80% relative humidity, and these conditions were supplied in an environmentally controlled growth chamber. About one gram of slow release fertiliser (Osmocoat N: P: K =14-13-13) was put onto the surface of the soil at the time of planting, the sizes of pots were 15 cm in height and 14 cm in width.

2.2 Bacterial strains and growth conditions

E. coli strain XL1-Blue MRF cells (Stratagene) and *A. tumefaciens* strain EHA105 (which was obtained from Dr. S. Gelvin, Purdue University, USA) were used in this work, bacteria were cultured in LB medium (the composition of LB was 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5 (w/v) NaCl, pH 7.0). Two types of this medium were used, LB liquid medium for plasmid DNA preparation and solid LB medium (solidified with 1.5% (w/v) of agar) for bacterial growth. The media were autoclaved at 121 °C for 20 min before using.

E. coli cultures were grown aerobically in solid media or LB broth at 37 °C with shaking at 200 rpm (platform shaker Innovation 2100) overnight, while *A. tumefaciens* cultures were grown under the same conditions except the temperature was 28 °C for 2-3 days. For the white-blue screening method a solid LB medium was prepared, followed by adding 25 µl of X-Gal (100 mg/ml in DMSO) and 100 µl of IPTG (100 mM) to each LB plate, then, spreading over the surface of the plates and left opened for 30 min to dry.

2.3 Plasmids

The plasmids used in this study are showed in Table 2.1; the maps of the plasmids are shown in Fig. 2.1.

2.4 Antibiotics

All antibiotics and their concentrations used in this work for growing and selection of bacteria are listed in Table 2.2.

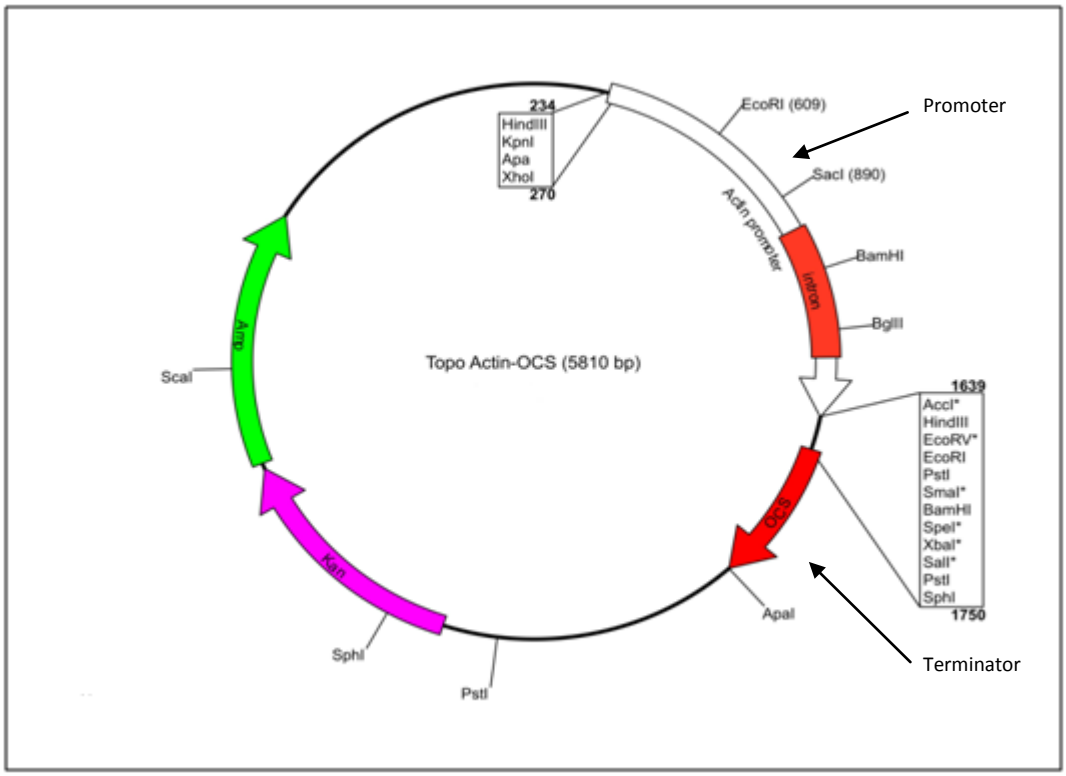
Table 2.1 Plasmids used in this work.

Plasmids	Description	Size/ Base pairs	Source
pActin-OCS	Contains pTopoactin-ocs	5810	Lab T23, HWU
WBvec.8	Binary vector with plant hygromycin resistance cassette	11280	Dr. H. Steinbiss, Max Planck Institute, Cologne, Germany (Wang *et al.*, 1998)
pBluescript(pflc111)-flbaf180a19	Contains cDNA for *HvMAPK4*	4284	Dr. K. Sato, Okayama University, Japan
p*Actin-HvMAPK4-OCS*	Contains ORF of *HvMAPK4*	6938	
WBVec.8- *Actin-HvMAPK4-OCS*	Contains ORF of *HvMAPK4*	13980	

Table2.2 Antibiotics used in this study (mg/l).

Antibiotics	Conc. mg/l	Plasmid/Bacteria
Ampicillin, Kanamycin	50	pTopoactin-ocs
Spectinomycin	50	pWBVec.8
Ampicillin	50	pBluescript
Rifampicin	25	*A. tumefaciens*
Timentin	160	Transformed barley calli
Hygromycin	50	

A-



B-

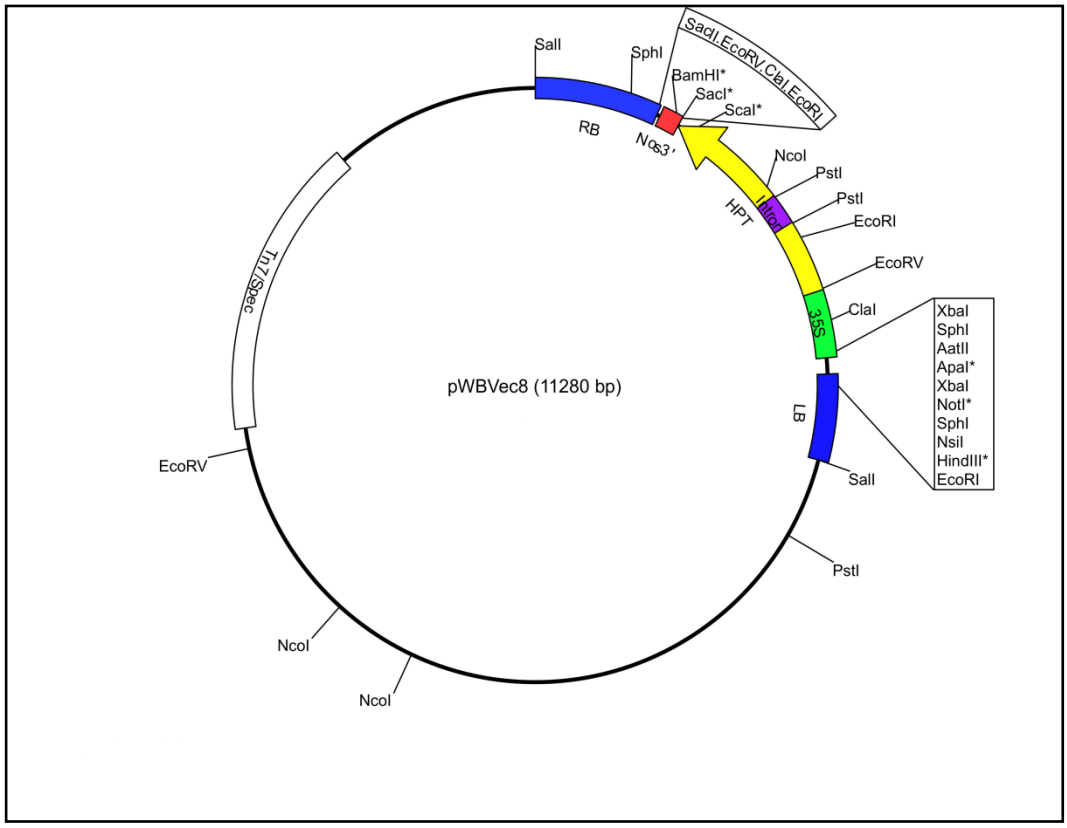


Figure 2.1 Plasmid maps

A. pActin-OCS (5810 bp).

B. pWBVec.8 (11280bp).

2.5 Storage conditions

All the plasmid DNA and PCR products were stored at -20 °C, the bacterial strains were stored at -70 °C in LB containing 15% (v/v) glycerol, and the bacterial cultures on LB plates were stored at 4 °C. All RNA containing solution (RNA, DIG-labelled RNA probes) were stored at -70 °C.

2.6 Sequence data and database search

An amino acid sequence alignment between all rice MAPK sequences and all publically available barley MAPK sequences was carried out using the ClustalW2 programme, which confirmed the identity of the barley homologue of the rice *OsMAPK4* gene (*HvMAPK4*). Dr. K. Sato, Okayama University, Japan was able to donate us full length cDNA barely clones encoding MAPK homologues, including *HvMAPK4*.

2.7 Chemical, enzymes and other materials

Suppliers of chemicals, reagents, enzymes and kits which were used in this work are listed in Table 2.3.

2.8 Centrifugation

Small sample volumes were centrifuged in 1.5 ml Eppendorf tubes in a Micro Centaur microfuge (MSE) device, the highest speed of MES is 13.000 rpm at room temperature, the large samples volumes were centrifuged in an Allegra, X-12R, Beckman Coulter, and Avanti, J-26 XD, Beckman Coulter machines at 4 °C at the stated speeds.

Table 2.3 The suppliers of chemicals, reagents, enzymes and kits

Suppliers	Chemicals, reagents, enzymes and kits
Duchefa Biochemie Melfords, Ipswich, UK	Murashige and Skoog medium (micro and macro elements)
Greiner Stonehouse, UK	1.5 ml, 15 ml and 50 ml polypropylene tubes, Petri dishes
Kodak Sigma, Dorset, UK	X-ray film developer and fixer solutions
MBI Fermentas Hanover. MD, USA	Restriction endonuclease enzymes, T4 DNA ligase, DNA and RNA markers
Melford Labs. Ipswich, UK	Agarose, bulk chemicals
Oxoid Basingstoke, UK	Agar, tryptone and yeast extract
QIAGEN West Sussex, UK	PCR DNA and Gel band purification kit.
Roche Lewes, UK	Anti-digoxigenin antibody conjugated to alkaline phosphatase, blocking reagent, positively charged nylon membrane and detection film.
Whatman Maidstone, UK	3 MM paper, filter paper

All chemicals, enzymes and other materials were handled and stored according to manufacturer's recommendations.

2.9 Images

In this study, pictures of the whole plant and callus were taken using a Nikon Coolpix S9 digital camera, while the agarose gel photos for both RNA and DNA were taken by using a UVP gel document system (Sony).

2.10 Recombinant DNA methods

2.10.1 Preparation of plasmid DNA

2.10.1.1 Preparation of plasmid DNA from *E. coli*

For mini-preparation of plasmid DNA an alkaline lysis method was used according to Birnboim (1983). 5 ml of LB broth plus the appropriate antibiotics was inoculated with a single colony of *E. coli* previously grown on LB solid medium overnight at 37 °C, the liquid culture was grown at the same conditions with shaking at 200 rpm, and 1.5 ml of this culture was centrifuged at 13000 rpm for 5 minutes. The supernatant was removed and the pellets were resuspended in 100 µl of GTE (50 mM glucose, 25 mM Tris-HCl, 10 mM EDTA at pH 8.0 with 1 µl of 10 mg/l of RNase) by vortexing, the samples were left for 5 min at room temperature. 200 µl of freshly prepared NaOH/SDS (0.2 M NaOH, 1% (v/v SDS) was added and mixed gently by inverting the tubes, and placed on ice for 5 min, 150 µl of 3 M potassium acetate (pH 4.8) solution was added and mixed by gentle inversion of the tubes. After 5 min on ice, the tubes were spun down for 3 min at 13000 rpm, the supernatant (containing the plasmid DNA) was removed into a fresh tube and mixed with 800 µl of 96% ethanol and allowed to incubate for 2 min at room temperature, after a centrifugation at 13000 rpm for 2 min the supernatant was discarded and the pellets washed in 1 ml of 70% ethanol, another centrifugation for 2 min at 13000 rpm was carried out, then the supernatant was discarded and the pellets dried and resuspended in 30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at -20 °C.

2.10.1.2 Preparation of plasmid DNA from *A. tumefaciens*

A single colony of *A. tumefaciens* (previously grown on LB plates for 2-3 days) was inoculated in 5 ml of LB broth containing an appropriate antibiotic and grown on a shaking platform at 200 rpm for 3 days. 1.5 ml of this culture was spun down at 13000 rpm for 3 min. The supernatant was removed and 3-4 cultures were combined in a total volume of 100 µl of ice cold lysis buffer (GTE solution supplemented with 4 mg/l lysozyme), vortexed and incubated at room temperature for 1 min, 200 µl of freshly prepared NaOH/SDS solution was added and mixed gently by shaking and incubated for 30 min at room temperature, 150 µl of sodium acetate (pH 4.8) was added, mixed and placed on ice for 5 min.

Samples were spun down at 13000 rpm, the supernatant was discarded and an equal volume of phenol: chloroform (1:1) was added and mixed, another centrifugation was carried out, and the top phase of the supernatant was transferred into a fresh tube. 1 ml of 96% ethanol was added and mixed gently, the mixture then centrifuged for 5 min, the supernatant was removed and the pellets were washed in 1 ml of 70% ethanol and spun down for 5 min, the supernatant was discarded and the pellet was dissolved in 50 µl of TE buffer. One µl of 10 mg/l of RNase was added and incubated at room temperature for 20 min.

2.10.2 Amplification of DNA by Polymerase chain reaction (PCR)

A standard 50 µl PCR reaction was used for all PCR reactions. The 50 µl of PCR reaction mixture was used in a 0.2 ml of polypropylene tube with the protocol:

DNA (approximately 100 ng for genomic DNA or 10 ng for plasmid DNA)

5 µl 10X polymerase buffer

8 µl dNTP (1.25mM)

0.5-1 µl polymerase (0.5 µl for *Pfu* and 1 µl for *Taq*)

1 µl of each forward and reverse primer (10 pmol/ µl)

The volume was completed to 50 µl by adding distilled water.

Primer stock solutions were prepared by reconstitution with the recommended amount of distilled water to get 100 pmol/µl as a final concentration. The thermal cycler used in this study was a Gene Amp® PCR system 2700, Applied Biosystems.

The PCR reaction started with an initial denaturation step at 94 °C for 5 min, before the addition of *Taq* DNA polymerase, the following steps were denaturation, annealing and extension, then extension step at 72 °C for 7 min, finally cooling and holding at 4 °C. (Fig 2.2).

Amplification of the MAP kinase contained in barley clone flbaf180a19 was carried out by PCR and primers which were chosen so that they would amplify the open reading frame (1128 bp) and introduce a suitable restriction site for *Xba*1 enzyme (**TCT AGA**) at both the 5' and 3' ends of the sequence to ease cloning later. The primer sequences and predicted sizes are shown in Table 2.4.

Table 2.4 Primers sequence and predicted size of PCR product.

All the primers were provided by MWG Biotech

Primer name	Primer sequence (5' ⟶ 3')	Predicted PCR product size (bp)
HvMAPK4-F	CCGTCTAGATGGCGATGATGGTGG (*Xba*I site)	1128
HvMAPK4-R	TCCTCTAGATCACATATTCACCATCCT(*Xba*I site)	
M13-F	GTTTTCCCAGTCACGAC	Dependent on cloned insert
M13-R	CAGGAAACAGCTATGAC	
Hyg-F	AAAAGTTCGACAGCGTCTCC	1068
Hyg-R	ATTTGTGTACGCCCGACAGT	
OCS-II	GAATGAACCGAAACCGGCGGTA	
HvPR1-F	GAGCAGGCCCATAGAAATCA	580
HvPR1-R	GGAATTCGAAGGTGCATGAG	
HvTub-F	TACCACCTCCCTGAGGTTTG	217
HvTub-R	CCATGCCTAGGGTCACACTT	

For *HvMAPK4* (F+R primers)

94 °C	94 °C	58 °C	72 °C	72 °C	4 °C
5 min	30 sec	30 sec	1min	7 min	Hold

30 cycles

For *HvTub* (F+R primers)

94 °C	94 °C	63.5 °C	72 °C	72 °C	4 °C
5 min	30 sec	30 sec	1min	7 min	hold

30 cycles

For M13 (F+R primers)

94 °C	94 °C	50 °C	72 °C	72 °C	4 °C
5 min	30 sec	30 sec	1 min	7 min	hold

30 cycles

For *HvPR1* (F+R primers)

94 °C	94 °C	52 °C	72 °C	72 °C	4 °C
5 min	30 sec	30 sec	30 sec	7 min	hold

35 cycles

For *Hyg* (F+R primers)

94 °C	94 °C	57 °C	72 °C	72 °C	4 °C
5 min	30 sec	1 min	1 min	7 min	hold

35 cycles

Figure 2.2 PCR cycles for different sets of primers.

2.10.3 Restriction Enzyme Digestion

For digestion of plasmid DNA, a total volume of 20 µl was used. Approximately 0.5-2 µg of undigested plasmid DNA were used for each digestion, and for each 1 µg of undigested plasmid DNA 10 units of restriction endonuclease was used. Reactions were incubated for 2 h at the appropriate incubation temperature.

Restriction enzymes, their buffer compositions and incubation temperature are listed in Table 2.5.

Table 2.5 Restriction enzymes, their buffers and incubation temperature

Enzyme	Buffer composition(10x)	° C
***Apa*I**	330 mM Tris-HCl, 660 mM K-acetate, 100 mM Mg-acetate, 5 mM DTT, pH 7.9	30
***Bam*HI**	10 mM Tris-HCl, 100 mM KCl, 5 mM $MgCl_2$, 0.02% Triton X-100, 0.1 mg/ml BSA, pH 8.0.	37
***Eco*RI**	50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 0.02% Triton X-100, 0.1 mg/ml BSA, pH 7.5.	37
***Eco*RV**	100 mM M Tris-HCl, 100 mM $MgCl_2$, 100 mM KCl, 1 mg/ml BSA, pH 8.5	
***Hind* III**	10 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, 0.1 mg/ml BSA, pH 8.5	37
***Pst*I**	50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA, pH 7.5	37
***Sal*I**	50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 0.1 mg/ml BSA, pH 7.5	37
***Sma*I**	33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate, 0.1mg/ml BSA, pH 7.9	30
***Spe*I**	100 mM Tris-HCl, 1 M NaCl, 120 mM $MgCl_2$, 10 mM DTT, pH 7.5	37
***Xba*I**	33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate,0.1 mg/ml BSA, pH 7.9	37

2.10.4 Agarose gel analysis of DNA fragments

The analysis and separation of DNA fragments were performed by agarose gel electrophoresis (1% w/v) using 0.5x TBE (10x stock solution TBE contains: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH8, H_2O to 1 litre) as a buffer supplemented with 1µl of ethidium bromide to a final concentration of 0.5 µg/ml in the gel. The DNA samples were mixed with 6x gel loading buffer (60 %(v/v) glycerol, 60 mM EDTA, 0.09% (w/v) xylene cyanol and 0.09% (w/v) bromophenol blue) and loaded onto the gel, a molecular weight of marker *λ Hind*III gives detectable fragments of: 23130, 9416, 6557, 4316, 2322, 2027 and 564 bp. The electrophoresis was carried out at 100V (RunOne-Electrophoresis cell-EmbiTec.) for 30 min, the DNA was visualized under UV illumination at 280 nm.

2.10.5 Agarose gel analysis of RNA fragments

RNA was separated by electrophoresis through 1% (w/v) agarose gel in 1x MOPS buffer (10x MOPS buffer contains: 200 mM MOPS, 50 mM sodium acetate, 10 mM EDTA at pH 7.0, autoclaved), containing 10 ml of 37% formaldehyde per 100 ml gel solution. RNA was mixed with two volumes of RNA loading buffer (contains: 50% (v/v) formamide, 16% (v/v) formaldehyde, 10% (v/v) of 10x MOPS buffer, 0.1 mg/ml ethidium bromide and 0.01% (w/v) bromophenol blue), then denatured at 70 °C for 10 min, rapid cooling on ice, the running buffer used was 1x MOPS and the electrophoresis was conducted at 80V.

2.10.6 Gel purification of DNA fragments

A QIAquick gel extraction kit protocol was used for the extraction of DNA fragments. The band of the important size was excised under the UV light with a clean scalpel, the gel slices were weighted and the instructions of the kit were followed.

2.10.7 Phenol: chloroform extraction

To remove protein from nucleic acid containing solutions, one volume of the DNA or RNA containing solution was mixed with one volume of 50:50 Tris-HCl (pH 8) saturated phenol: chloroform for DNA or with water saturated phenol-chloroform for RNA, in a 1.5 ml microfuge tube, and then vortexed, centrifuged for 10 min at 13000 rpm. The top phase of the supernatant (which contains the nucleic acid) was removed into a new microfuge tube and mixed with an equal volume of chloroform to remove any residual phenol, another centrifugation for 10 min at 13000 rpm was carried out, and the upper phase was transferred into a fresh tube and used for ethanol precipitation.

2.10.8 Ethanol precipitation

The DNA or RNA was precipitated from the solutions by the addition of 0.1 volume of 3 M sodium acetate at pH 5.2, and 2.5 volumes of 96% (v/v) of ethanol. The samples then mixed and incubated for 30 min at -20 °C, a centrifugation for 15 min at 13000 rpm was carried out, the supernatant was removed and the pellets washed with 1 ml of 70% (v/v) ethanol, another centrifugation for 10 min at 13000 rpm was carried out and the supernatant was discarded and the pellets were dried and resuspended in TE for DNA or DEPC-dH_2O for RNA at the desire volume.

2.10.9 Dephosphorylation of DNA

To prevent recirculation of plasmid DNA during the ligation during cloning, one unit of shrimp alkaline phosphatase (SAP) was added to the digested fragments with 1x SAP buffer (10x buffer is 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 9), mixed gently and incubated at 37 °C for 30 min, followed by 10 min at 70 °C to inactivate the phosphatase.

2.10.10 Ligation of DNA fragments

DNA fragments were ligated in a total volume of 20 µl at a molar ratio of 1:3 vector: insert for linearised vector and insert. The vector and insert were added together to a sterile microfuge tube along with 2 µl 10x ligation buffer (0.4 mM Tris-HCl, 0.1 mM $MgCl_2$, 0.1 M DTT, 5 mM ATP, pH 7.8) and 5 units of T4 DNA ligase. Samples were incubated at 37 ° C for 4 h at room temperature, followed by inactivation of ligase by heating the mixture at 75 °C for 15 min.

2.10.11 Preparation of competent cells for transformation

2.10.11.1 Preparation of *E.coli* competent cells for transformation

The procedure of Inoue *et al.* (1990) was followed to prepare the competent *E. coli* cells. 5 ml of LB broth medium contains 100 µl of 1M $MgSO_4$ was inoculated with single colony of the *E. coli* strain XL-1 Blue and incubated at 37 °C overnight on the platform shaker (200 rpm). This culture was used to inoculate 250 ml of LB broth and incubated at room temperature with good aeration until the OD_{600} reached 0.4-0.6. Subsequently the culture was transferred into a sterile bottle and placed on ice for 10 min, spun down at 3000 rpm for 10 min at 4 °C, the pellets resuspended gently in 80 ml cold transformation buffer (10 mM Pipes-HCl, pH 6.7, 15 mM $CaCl_2$, 250 mM KCl and 55 mM $MnCl_2$) and spun down at 3000 rpm for 10 min at 4 °C. The pellets were resuspended in 20 ml cold transformation buffer with 1.5 ml DMSO, placed on ice for 10 min and dispensed into 200 µl aliquots in ice cold sterile microfuge tubes and kept at -70 °C.

2.10.11.2 Preparation of *A. tumefaciens* competent cells for transformation

A. tumefaciens strain EHA105 was grown on LB solid medium at 28 °C for 3 days, a single colony of this culture was inoculated into 5 ml LB broth containing Rifampcin at 25 mg/l, and kept at 28 °C overnight. 2 ml of this culture was added to 50 ml LB broth in a 250-ml flask and shaken vigorously (250 rpm) at 28 °C until the culture reached an OD_{600} of 0.5-1.0, after that, the culture was chilled on ice and centrifuged at 3000 g for 5 min at 4 °C. The supernatant was removed and the pellets were resuspended in 1 ml of ice-cold 20 mM $CaCl_2$ solution and dispensed in 100 µl aliquots into prechilled Eppendorf tubes and kept at -70 °C.

2.10.12 Plasmid construction

A full-length cDNA clone of barley *HvMAPK4* (plasmid flbaf180a19) was used to amplify and clone the ORF of *HvMAPK4* by using PCR primers with *Xba*I restriction sites at both 5' and 3' ends. The PCR product was digested with *Xba*I and inserted into the *Xba*I site of pActin-OCS (5.8 kbp: this vector contains the promoter of the rice actin gene including the first intron: see plasmid map, Fig. 2.1).

In this way, *HvMAPK4* could be cloned in both orientations, giving both sense and antisense constructs. The insert *Actin-HvMAPK4-OCS* was excised with the *Apa*I enzyme and inserted into pWBVec.8 which was previously digested with *Apa*I to obtain overexpression and antisense constructs for transfer into barley, then transformed into *A. tumefaciens*. As a control, empty binary vector (pWBVec.8) was transformed into *A. tumefaciens*. All constructs were checked by digestion with the restriction enzyme *Hind*III and also by PCR (for overexpression construct the primers: forward *HvMAPK4* and reverse OCS were used, while for antisense construct the primers: reverse *HvMAPK4* and reverse OCS were used) to confirm the orientation of constructs. The ORF of the overexpression construct was fully sequenced in order to confirm that no errors had occurred during PCR or cloning. The transformations into *E. coli* and *A. tumefaciens* were conducted according to the protocols 2.10.13 and 2.10.14, respectively.

2.10.13 Heat shock transformation of *E. coli*

An appropriate volume of plasmid DNA (10 µl of a ligation or 10 ng supercoiled DNA) was added to 200 µl of competent cells in 1.5 ml-microfuge tube (stored at -70 °C and subsequently thawed on ice) and mixed gently. The samples were placed on ice for 30 min, then the cells were heat-shocked for 1 min at 42 °C in a water bath, then placed on ice for 2 min. 1ml of LB was added to the samples (without addition of any antibiotics) and incubated at 37 °C for 1 h. The cells were pelleted by centrifugation at 6000 rpm for 3 min in a microfuge tube, most of the supernatant was removed, and the cells were resuspended and spread onto LB plates supplemented with appropriate antibiotics, and incubated at 37 °C overnight.

2.10.14 *A. tumefaciens* transformation

A. tumefaciens transformation was performed using a modified free-thaw method (Hofgen and Willmitzer, 1988). An appropriate volume (about 1 µg in weight) of plasmid DNA was added to the competent cells in a 1.5 ml microfuge tube and incubated at 37 °C for 5 min, one ml of LB medium was added to the sample and incubated at 28 °C for 4 h with gentle shaking, centrifugation was carried out for 1 min at 13000 rpm. The supernatant was removed and 100 µl of LB medium was added to resuspend the pellets. The cells were then spread on LB plates supplemented with appropriate antibiotics; incubation was carried out at 28 °C for 3 days.

2.10.15 Preparation of DIG-labelled *HvMAPK4* DNA probe

A standard 50 µl PCR reaction (see section 2.10.2) with both forward and reverse *HvMAPK4* primers was carried out on a plasmid containing the ORF of *HvMAPK4* fragment (10-20 ng) for preparation of the probe. 2 µl of DIG 11-dUTP (Roche) was added to the reaction. An aliquot of the PCR product was run on the agarose gel together with the same PCR product without incorporated DIG 11-dUTP to check the probe and if a size shift between two fragments was seen, then the labelling was assumed successful.

2.10.16 Preparation of DIG-labelled RNA probe

M13 forward and reverse primers were used to amplify the desired DNA fragment from a cloning vector through a standard PCR reaction (see section 2.10.2). According to the orientation of cloned insert, the T3 or T7 RNA polymerase was used to produce a digoxigenin labelled antisense single stranded RNA from the PCR product, using the nested viral promoter.

The probe synthesis reaction contained about 200-500 ng of purified PCR product, 2 µl 10x transcription buffer, 2 µl 10x digoxigenin RNA labelling mix (Roche), 1 µl RNA polymerase, 30 mM DTT, 0.5 µl RNase inhibitor, and a final total volume of 20 µl using DEPC-treated dH_2O. The reaction was incubated at 37 °C for 2 h, 2 µl of the reaction was run on the gel together with high range RNA ladder (Fermentas) which gives a detectable range of fragments 6000, 4000, 3000, 2000, 1500, 1000, 500 and 200 bp for quality and quantity assessment.

Two RNA probes were prepared and used in this study, which were *HvMAPK4* (1128bp) to check the expression of barley MAP Kinase 4, and *PR1* (580 bp) as a pathogenesis-related protein 1 to detect the expression of this gene in plant-pathogen interaction.

2.10.17 DEPC-treatment of solution

All solutions (other than Tris buffer) to be used with RNA were treated with DEPC to remove any RNase present, 100 µl of DEPC was added to 1 litre of solution and the mixture stirred for 1 h at room temperature, then, any remaining DEPC was removed by autoclaving the solution.

2.10.18 Isolation of barley genomic DNA

The protocol of Dellaporta *et al.* (1983) was followed to extract the genomic DNA from the barley plant. A 1 cm^2 of a leaf blade was removed into a 1.5 ml microfuge tube and ground with a small pestle in 750 µl of extraction buffer (contains: 50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, 10 mM 2-mercaptoethanol, pH 8.0), and the mixture was incubated at 65 °C for 10 min, 200 µl of 5 M potassium acetate (pH 4.8) was added and placed on ice for 20 min, following which a centrifugation was carried out at 13000 rpm for 10 min. The supernatant was removed into a fresh tube and an equal volume of isopropanol was added and mixed to precipitate the DNA.

The mixture was spun down for 2 min at 13000 rpm, the supernatant was discarded and the pellets was washed in 500 µl of 70% ethanol and centrifuged for 10 min, then the supernatant was removed and the pellets were resuspended in 30 µl of TE buffer.

2.10.19 Isolation of total RNA from barley leaves

The protocol of Cathala *et al.* (1983) was followed to isolate the total RNA from barley. On day one, 0.5 g (as fresh weight) of barley leaves was ground in liquid nitrogen to a fine powder, using a pre-chilled pestle and mortar, then transferred into a 1.5 ml sterilised microfuge tube containing 500 REB buffer (contains: 25 mM Tris-HCl, 25 mM EDTA, 75 mM NaCl, 1% (w/v) SDS, pH 8.0, autoclaved) and vortexed well, and 500 µl of water-saturated phenol/chloroform (50:50 v/v) was added and vortexed. A centrifugation for 5 min at 13000 rpm was carried out, the upper aqueous layer was transferred onto a new tube containing 500 µl of water-saturated phenol/chloroform, the tube was vortexed and centrifuged as mentioned before.

The top layer was transferred into a fresh tube containing 500 µl of chloroform, followed by vortexing and centrifugation as before. The upper layer was removed into fresh tube, and 0.25 volume of DEPC-treated 10 M LiCl was added very slowly and gently mixed and kept overnight at –20 °C for precipitation. On day two, a centrifugation at 13000 rpm for 30 min was carried out, the supernatant was discarded and the pellet was dried and resuspended in 100 µl of DEPC-treated dH_2O. The ethanol precipitation for RNA was done as in section 2.10.8, the final volume for dissolving RNA pellets was 20 µl in DEPC-treated dH_2O.

The concentration of RNA was measured spectrophotometrically at optical density (OD) 260 nm. 1 µl of the RNA was diluted in 400 µl of TE buffer and vortexed, then transferred into a quartz cuvette with 10 mm path length, the absorbance at OD_{260} was recorded and used to calculate the quantity of RNA. An OD_{260} reading of 1 in a quartz cuvette with a 1 cm path length corresponds to a concentration of 40 µg/ml of RNA.

2.10.20 Colony hybridisation of transformed *E. coli*

A colony hybridisation after transformation of *E. coli* competent cells was carried out according to Buluwela *et al.* (1989) to detect the positive colonies which contain the insert *HvMAPK4*. After growing of the bacteria on LB solid medium, a nylon membrane (Hybond-N, Amersham) was placed onto the plate, marking the positions of the nylon membrane, then the filter was removed and the plate was incubated again overnight at 37 °C to give the colonies a chance to resume their growth. The membrane was placed on a Whatman 3 MM papers that was pre-wetted with 2x SSC, the membrane placed with the colonies face up.

The membrane with filter was placed in a microwave oven and heated for 2.5 min at 650 Watts to lyse the cells. The colonies were then prehybridised for 1 h in 10 ml of DIG Easy Hyb buffer (Roche) at 65 °C; a rotating roller bottle in a hybridisation oven (Hybaid) was used. The DIG-labelled probe of *HvMAPK4* was denatured by boiling in hybridisation solution for 5 min, followed by rapid cooling on ice.

The prehybridisation solution was discarded and the Easy Hyb buffer (10 ml) containing denatured DNA probe was added to the membrane (about 25 ng of the probe for each 1 ml of the buffer) and hybridised overnight at 65 °C. After hybridisation, the membrane was washed once in 2x SSC, 0.1% (w/v) SDS at 65 °C for 20 min, then twice with 0.2x SSC, 0.1% (w/v) SDS at 65 °C. The membrane was then washed in 10 ml of DIG 1 buffer (0.1 mM Tris-HCl, 1 M $NaCl_2$, 0.2 % (v/v) Tween 20 at pH 8.5) and incubated in blocking solution (0.5 % (w/v) blocking reagent in DIG 1 buffer) for 1 h at room temperature with gentle shaking.

Anti-DIG antibody conjugated to alkaline phosphatase was diluted 1:20,000 in blocking buffer and the membrane incubated in this solution for 30 min at room temperature with gentle shaking. The detection of the signal was carried out colorimetrically. A colour solution (which contains: 35 µl of 5-bromo-4-chloro-3-indolyl phosphate and 45 µl of nitrotetrazalium blue chloride) was added and the mixture was incubated at 37 °C in the dark, then membrane washed in dH_2O and dried to stop the reaction, the positive colonies appeared in a purple colour.

2.10.21 Northern blotting

An amount of 10 or 20 µg of total RNA was separated on a denaturing agarose gel (see section 2.10.5), after fractionation, a photograph was taken under UV light, then the gel rinsed in 20 x SSC (3 M NaCl, 0.3 M sodium acetate, pH 7.0) for 10 min to remove any formaldehyde. The RNA was then blotted onto a nylon membrane. The blotting stack consisted of 2 pieces of Whatman 3 MM paper which were previously pre-wetted with 20x SSC and placed on the top of a thick stack of paper towels, a piece of positively charged nylon membrane the same size of the gel (which was pre-wetted with dH_2O and then with 20x SSC) placed on the top of Whatman 3MM paper. The slots of the gel were cut off and placed on the top of the membrane; another 2 pieces of Whatman paper were used to cover the gel. A paper bridge was constructed to bring the 20x SSC from a reservoir by using two long pieces of Whatman 3MM paper. Cling film was used to cover the blot and reduce the evaporation during the transfer.

The blotting took place between 4-6 h on the bench at room temperature, and after the blotting was completed, the RNA was fixed onto the membrane by using a UV crosslinker (UVC-508, Anachem: 120,000 µJ of UV). Pre-hybridisation was carried out for the RNA blot for 30 min with 10 ml of hybridisation buffer (6 M urea, 6x SSC, 1% (w/v) SDS and 100 mM Tris-HCl, pH 7.0), once the pre-hybridisation was finished, the DIG-labelled antisense RNA probe (100 ng/ml of hybridisation buffer) was denatured at 100 °C for 10 min and used for hybridisation of the blot overnight at 65 °C.

After the hybridization was completed, the blot was washed once in 2x SSC, 0.1% (w/v) SDS (low stringency wash) for 1 h and twice in 0.2x SSC, 0.1% (w/v) SDS (high stringency wash) for 20 min for each at 65 °C. The membrane was briefly washed in DIG 1 buffer (0.1 M Tris-HCl, 1 M NaCl, 0.2% (v/v) Tween 20 at pH 8.5). The blot was blocked in blocking solution (0.5% blocking reagent Hammerstein casein, 0.1M

maleic acid, 350 mM NaOH, 1M NaCl, 0.2% Tween, pH 8.5, DEPC-treated and autoclaved) for 1 h at room temperature.

Anti-DIG antibody conjugated to alkaline phosphatase (Roche) was diluted 1: 20,000 in blocking buffer and then used to incubate the membrane for 30 min at room temperature. The membrane then washed four times for 10 min each in large volume of DIG1, finally rinsed in DIG 4 as detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 2 min, then the substrate solution (CDP Star, Sigma) was added to the membrane. X-ray film was used to detect the resulting chemiluminescent signal.

2.10.22 Development of X-ray film

Developer and fixer solutions (Kodak) were diluted in dH_2O according to the manufacturer's instructions; all steps of development were carried out at dark room using red safety light. The exposed X-ray film was placed firstly in developer solution with gently agitation, and then rinsed in water before being placed in fixer solution, finally the developed X-ray film was rinsed in water and let to dry, the exposure time of the X-ray film was between 30 min to 3 h depending on the intensity of the signal.

2.11 Barley transformation

2.11.1 Growth of *A. tumefaciens*

A. tumefaciens strain EHA105 containing a pWBVec-8 derived binary vector was grown on LB plates contains 25 and 50 mg/l of rifampcin and spectinomycin, respectively, and incubated for 3 d at 28 °C. A single colony of this culture was inoculated into a 5 ml of LB broth contains both mentioned antibiotics and incubated at 28 °C overnight, and 400 µl of this broth culture was used for barley transformation.

2.11.2 Preparation of Barley Immature Embryos (IEs)

Barley cultivar Golden Promise were grown from seeds in a growth chamber (see section 2.1 for growth conditions), and spikes of healthy looking plants were collected two weeks after pollination. The awns were removed from the spikes and the seeds surface sterilised by immersing in 70% ethanol for 1 min, then in 1% of sodium hypochlorite for 20 min, and rinsed four times in dH_2O. All steps were carried out in a laminar flow air cabinet to ensure sterility. The immature embryos (1.5-2.5 mm in diameter) were then dissected from the young caryopsis with a scalpel using a dissecting microscope, the embryonic axis was removed and the remaining embryo placed scutellum side down on solid callus induction medium CIM (Tingay *et al.*, 1997; Bartlett *et al.*, 2008), the composition of CIM- $CuSO_4$ was:

4.3 g/l Murashige and Skoog plant salts

30 g/l Maltose.

690 mg/l Proline.

250 mg/l Myo-inositol.

1 g/l Casein hydrolysate.

1 mg/l Thiamine HCl.

1.25 mg/l $CuSO_4.5H_2O$.

10 mM MES to pH 5.8.

3.9 g/l Phytagel.

2.5 mg/l Dicamba .

2.11.3 Barley transformation

The transformation was done in the same day as embryo preparation. The prepared immature embryos (IEs) of barley were inoculated with *A. tumefaciens* according to Tingay *et al.* (1997). A small drop (1-2 µl) of *A. tumefaciens* suspension was added into each embryo on the side where the axis previously was, placed scutellum side down on CIM-$CuSO_4$ plates and left open for 1 h in the laminar flow air cabinet to remove any excess liquid, then the IEs were dragged across the surface of the medium and then removed onto new CIM- $CuSO_4$ plates (25 embryos for each plate), and incubated in the dark at 24 °C for three days as a co-cultivation period. The overgrowth of *Agrobacterium* was monitored during the co-cultivation period, and in case of overgrowth, the infected callus was washed with Timentin (160 mg/l) for 30 sec and twice with dH_2O.

2.11.4 Selection of transformed plants

The protocol of Bartlett *et al.* (2008) was followed with some modification. After the co-cultivation period, the IEs were removed onto CIM- $CuSO_4$ supplemented with 50 mg/l and 160 mg/l of hygromycin and Timentin, respectively, and incubated in the dark for 6 weeks at 24 °C (selection stage). The IEs were transferred into fresh CIM-CuSO4 plates every two weeks during selection stage. After selection stage, callus had developed and this material was transferred onto plates with regeneration medium (RGM): which contains: 2.7 g/l Murashige and Skoog plant salts, 20 g/l maltose, 165 mg/l NH_4NO_3, 750 mg/l glutamine, 100 mg/l myo-inositol, 0.4 mg/l thiamine HCl, 0.1 mg/l 6-benzylaminopurine (6BA), 1.25 mg/l $CuSO_4.5H_2O$, 3.9 g/l Phytagel), and supplemented with 25 mg/l and 160 mg/l of hygromycin and Timentin, respectively, these plates were incubated at 24 °C for 4-6 weeks in full light (60 μEm^{-1} s^{-1}, 16 h light and 8 h dark cycles). Once the regenerated plants had shoots of 2-3 cm in length, they were transferred into cultures tubes which contained rooting media (CIM) without any additives, but still containing the antibiotics for selection. The incubation was carried out at the same conditions as for RGM plates for 2-3 weeks; the transformed developed plants with strong root system were transferred into soil and grown under the same conditions as the donor plants (section 2.1).

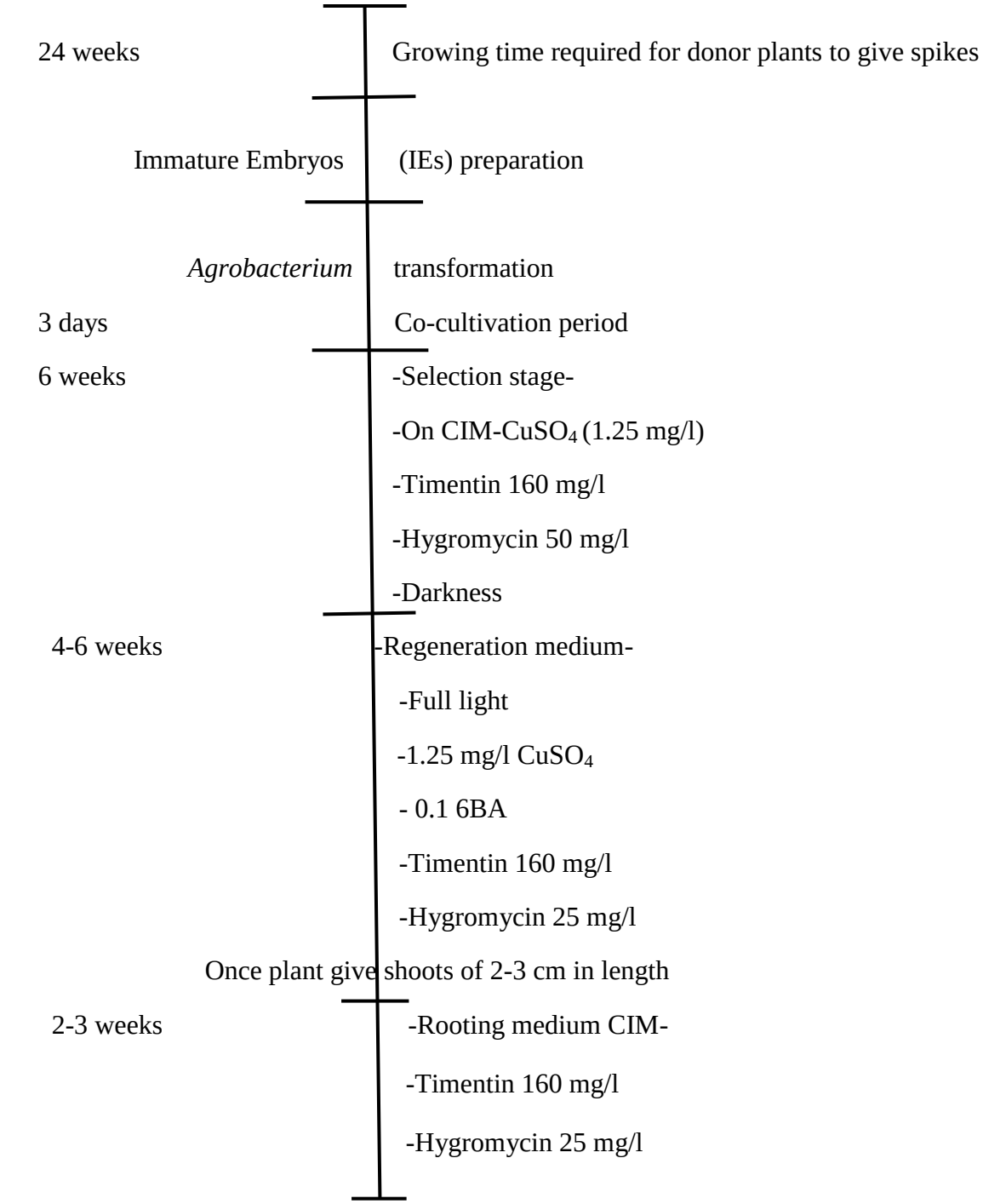


Figure 2.3 Time schedule for barley transformation.

2.12 Fungal infection assay

2.12.1 Fungal strain and growth conditions

The wildtype strain Guy-11 of *Magnaporthe oryzae* was obtained from Dr. N. Talbot, Exeter University, UK and cultured on CM plates (complete medium) which contained: 50 ml 20x nitrate salts, 1 ml 1000x trace elements, 1 ml vitamin solution, 10 g D-glucose, 2 g peptone, 1 g yeast extract and 1 g casamino acid, and 15 g of agar for solid medium.

The chemical composition of 20x nitrate salts (1 litre) is:

120 g $NaNO_3$

10.4 g KCl

10.4 g $MgSO_4.7H_2O$

30.4 g KH_2PO_4

The 1000x trace elements contains:

80 ml dH_2O

2.2 g $ZnSO_4.7H_2O$

1.1 g H_3BO_3

0.5 g $MnCl_2.4H_2O$

0.5 g $FeSO_4.7H_2O$

0.17 g $CoCl_2.6H_2O$

0.16 g $CuSO_4.5H_2O$

0.15 g $Na_2MoO_4.2H_2O$

5 g Na_4EDTA, pH6.5.

The vitamin solution (in 100 ml of dH2O) contains:

0.01 g Biotin

0.01 g Pyridoxine

0.01 g Thiamine

0.01 g Riboflavin

0.01 g p-aminobenzoic acid

0.01 g Nicotinic acid

These plates were kept at 27 °C with 16 h light and 8 h night cycles for 2 weeks; conidia were harvested from these cultures by rinsing with dH_2O and filtering through layers of gauze, then these suspensions were diluted 1:1 (v/v) with 0.1% (w/v) gelatin, 0.05% (v/v) Tween 20 as a spraying solution at a concentration 2x 10^5 conidia per ml (Zellerhoff *et al.*, 2006).

2.12.2 Plant material, inoculation and infection assay

The spring barley cultivar Golden Promise was used in this experiment, the plants were grown in a growth chamber at 18 °C with 16 h light and 8 h night cycles, primary leaves of 7 days old plants were spraying with conidia according to Zellerhoff *et al.* (2006), the inoculation process was done inside plastic covers to maintain high level of humidity which is required for the pathogen, the inoculated plants were incubated at 26 °C in the dark for 24 h, after that, the plant were incubated in normal growth conditions (18 °C with 16 h light and 8 h night cycles).

Disease assessment was performed 7 days post-inoculation, the lesion number and size were measured on the youngest leaf of infected barley and the symptoms were scored according to a scale of five landmark lesion types made by Valent *et al.* (1991).

The disease scales were:

1-Uniform dark brown pinpoint lesion without visible centres, the lesion diameter is 0.5 mm.

2-Small lesion with distinct tan centres surrounded by darker brown margins, the lesion diameter is 1mm.

3-Small eye spot lesion, the lesion diameter is 2 mm.

4-Intermediate size eyespot lesion, the lesion diameter is 3-4 mm.

5-Large eyespot lesions that attain the maximum size, the lesion diameter is 5 mm.

Type 1 is considered non pathogenic, while types 2, 3, 4 and 5 are considered pathogenic (Fig. 2.4).

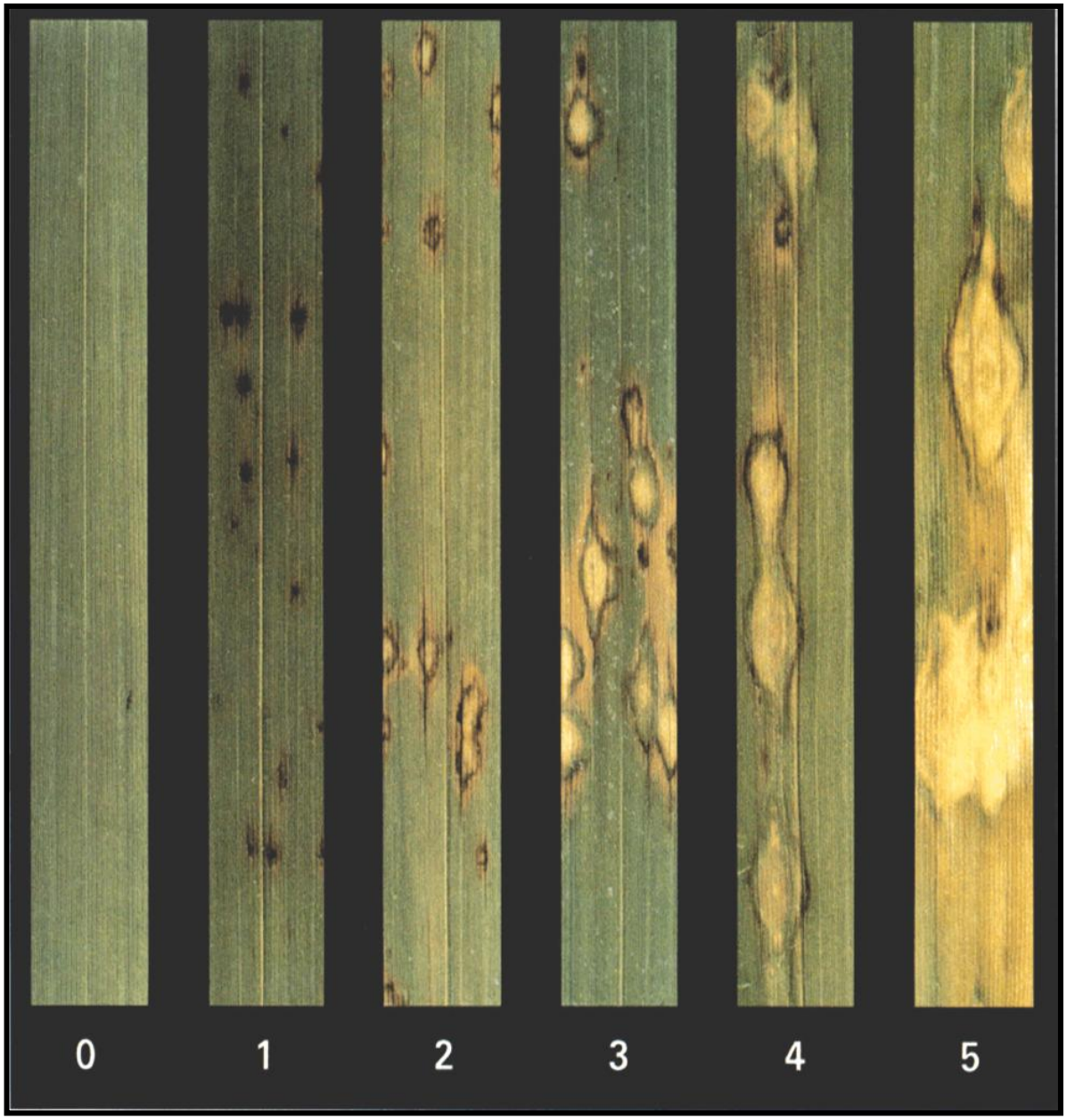


Figure 2.4 Leaf segments showing standard lesion types.

From: Valent *et al.* (1991).

2.13 Measurement of endogenous plant hormones

The leaves of barley plants (for wildtype and overexpression and antisense transgenic plants) were collected according to the time course: 0, 24 and 48 h post-inoculation with the blast pathogen, and rapidly frozen in liquid nitrogen after weighing, and plant phytohormones were measured.

2.13.1 Salicylic acid measurement.

Salicylic acid measurements were performed according the procedure of Engelberth *et al.* (2003) and Lee *et al.* (2004). Briefly, 250 mg of barley leaves were frozen and ground in liquid nitrogen, then homogenized in 4 ml of 70 mM potassium dihydrogen orthophosphate, pH 7.4. Propyl paraben was added as internal standard to give 5 ng in 2 µl. Samples were sonicated for 20 min and kept overnight in the fridge. On day two, another sonication for 5 min was done, and then centrifuged at 6000 rpm for 15 min, the supernatant was removed into a fresh tube, and the pellets was dissolved in 2 ml of 70 mM of potassium phosphate and centrifuged again.

The supernatants were pooled and passed through a 1 µM PTFE disc filter (Supelco), and then the filter was rinsed in 1 ml of water. 1 ml of 3 M HCl was added and mixed, 2 ml of chloroform was added and the mixture vortexed for 2 min and centrifuged for 15 min at 4000 rpm. The lower layer was removed onto a clean tube, and the chloroform extraction was repeated. Pooled extract were evaporated to dryness, the residues were dissolved in 50 µl of methanol, samples were methylated by addition of 5 µl of methprep1 and incubated overnight at room temperature, 2 µl of sample was used to analyse salicylic acid by GC/MS: gas chromatography- mass spectrometry.

2.13.2 Jasmonic acid measurement.

Jasmonic acid measurements were performed according the procedure of Engelberth *et al.* (2003) and Lee *et al.* (2004). Briefly, 250 mg of barley leaves were frozen and ground in liquid nitrogen, then homogenized in 4 ml of acetone: 50 mM citric acid (70:30). Dihydrojasmonic acid was added as internal standard, in order to give 2 ng in 2 µl when finally injected into the GC/MS. Samples were sonicated for 20 min and kept overnight at room temperature. On the day two, another sonication was done for 5 min, and then centrifuged at 6000 rpm for 15 min, supernatant was removed into a clean tube and the pellets dissolved in 2 ml of 50 mM of citric acid. Another centrifugation was repeated and the supernatants were pooled.

Pooled supernatants were passed through a 1 µM PTEF disc filter (Supelco) and extracted with 5 ml of diethyl ether and vortexing for 2 min. Pooled extracts were applied to a solid phase extraction cartridge (Varian Bond Elute NH2) containing 500 mg of packaging. The cartridges were rinsed in 10 ml of chloroform: isopropanol (2:1), the jasmonic acid and internal standard were eluted with 10 ml of diethyl ether: acetic acid (98:2). Solvents were evaporated under a gentle air stream and the residues were dissolved in 50 µl of methanol, samples were methylated with 5 µl of methprep1, and incubated for 20 min at room temperature. 2 µl of sample was used for jasmonic acid analyze by GC/MS: gas chromatography- mass spectrometry.

2.13.3 Ethylene measurement.

Ethylene level were measured as described by Locke *et al.* (2000), a weighed amount of fresh barley leaves (0.5 mg) were sealed into 22 ml glass tubes with 1 ml water, and incubated at 25 °C for 24 h, 1 ml of headspace gas was sampled from each tube and ethylene content was determined by gas chromatography system (Hewlett-Packard 5980 series II) equipped with Quadrex BTR-CW 50 meter length, 0.32 mm diameter column, run at 80 °C, between 1 and 10 ppm of ethylene were used as standard.

2.14 Hydrogen peroxide measurement.

2.14.1 Standardization of hydrogen peroxide

To standardize hydrogen peroxide, the procedure of Zhou *et al.* (2006) was followed. A 30% solution of H_2O_2 was diluted to 10 mM in 50 mM phosphate buffer at pH 7.0, the diluted solution was standardized by its absorbance at 240 nm, and measuring against a blank from which H_2O_2 had been removed by addition of catalase, the absorbance due to catalase addition was corrected for with the blank of catalase alone in the buffer.

2.14.2 Determination of hydrogen peroxide

The hydrogen peroxide was measured spectrophotometrically as described in Zhou *et al.* (2006), 0.5 g of barley fresh leaves were frozen and ground in liquid nitrogen and homogenized with 5 ml of 5% trichloroacectic acid and 0.15 g of activated charcoal, the mixtures were removed into a clean tube and centrifuged at 10,000 g for 20 min at 4 °C. The supernatants were removed into 50 ml tubes. The pH was adjusted to 8.4 with 17 M ammonia solution, the mixture then passed through a Millipore type A 0.45 µM filter. 1 ml of this sample was mixed with 1 ml of colorimetric reagent (contains: 10 mg 4-aminoantipyrine, 10 mg phenol and 5 mg peroxide, dissolved in 50 ml of 100 mM of acetic acid buffer, pH 5.6), and incubated for 10 min at room temperature. The absorbance at 505 nm was determined spectrophotometrically by using a UV-1650PC Shimadzu spectrophotometer.

2.15 Catalase activity.

Catalase activity was determined according to Vanacker *et al.* (2000), 1 g of barley fresh leaves were frozen and ground in liquid nitrogen, and extracted in 0.1 M KH_2PO_4/KOH, pH 7.4 and 30 mM DTT. The mixtures were centrifuged at 13000 rpm for 3min, the samples then kept on ice until the assay. Catalase was assayed polargraphically at 20 °C with a dissolve oxygen meter (OXi 315i) and a liquid phase oxygen electrode (DurOX 325). Catalase (from bovine liver, Sigma) was used to calibrate the electrode. Total extractable catalase activity was determined via O_2 evolution upon the addition of 0.5 M H_2O_2 to a reaction medium contains 100 mM HEPES/KOH, pH 7.4. One unit of catalase activity was defined as the quantity of catalase that would liberate 1 µM of O_2 in min.

2.16 *In vitro* study of salicylic acid and hydrogen peroxide effect on the growth of *M. oryzae*.

Different concentrations of salicylic acid and hydrogen peroxide were selected to study their direct effect on the growth of pathogen. For salicylic acid the tested concentrations were 72.5, 145, 290, 435 and 580 mM, while for hydrogen peroxide were 5, 10 and 50 mM.

2.16.1 Measurement of pathogen growth.

5 mm agar plugs taken from 7 day old culture of *M. oryzae* grown on CM plates were inoculated on the centre of CM plates alone as a control and with SA and H_2O_2 concentrations and incubated for 10 days at 27 °C with 16 h light and 8 h night cycles. Colony diameters were measured by taking the average of three directions on each petri plate. The effects of salicylic acid and hydrogen peroxide on mycelial growth were calculated as the formula (Yao and Tian, 2005):

2.16.2 Measurement of biomass production

5 mm agar plugs taken from 7 day old culture of *M. oryzae* grown on CM plates were used to inoculate 100 ml conical flasks filled with 50 ml of CM broth alone as a control and with salicylic acid and hydrogen peroxide concentrations. Cultures were incubated in a rotary shaker (170 rpm) at the same growth conditions as in section 2.15.1 for 10 days, then, the fungal biomass (dry weight) was measured after filtration and drying at 80 °C for 12 h. The inhibitory effects were calculated by following the formula in 2.16.1.

2.17 Statistics

All experiments were performed with three biological replicates. The results presented as means ± SD (standard deviation of the mean). SD was measured as the square root of its variance and used in this study to show the variation among replicates, the low SD indicates that the data are very close to the mean. The results were statistically analysed by one-way analysis of variance (ANOVA). All statistical analyses were performed with Minitab version 15 statistical software. Differences were considered significant when $P < 0.05$. Tukey's test was applied when one way ANOVA revealed significant differences. The data were plotted by using Microsoft Office Excel 2007.

CHAPTER 3 RESULTS

3.1 *HvMAPK4* sequence and amino acid composition

The publically available data for barley MAPK amino acid sequences was compared with the 17 rice MAPK amino acids sequences using ClustalW. A phylogenetic tree showed the relationship of barley clones and rice MAPKs. This elucidated which of the barley clones showed closest homology to *OsMAPK4* (Fig. 3.1, see Appendix 5.1 for full-length cDNA of the barley clone for *HvMAPK4*). Based on the phylogenetic tree and the published papers related to rice MAPKs and diseases resistance, the barley clone with the Genebank accession number AK252980 which is the barley homologue of *OsMAPK4* was selected for further study; thus, barley clone AK2525980 will be named *HvMAPK4*.

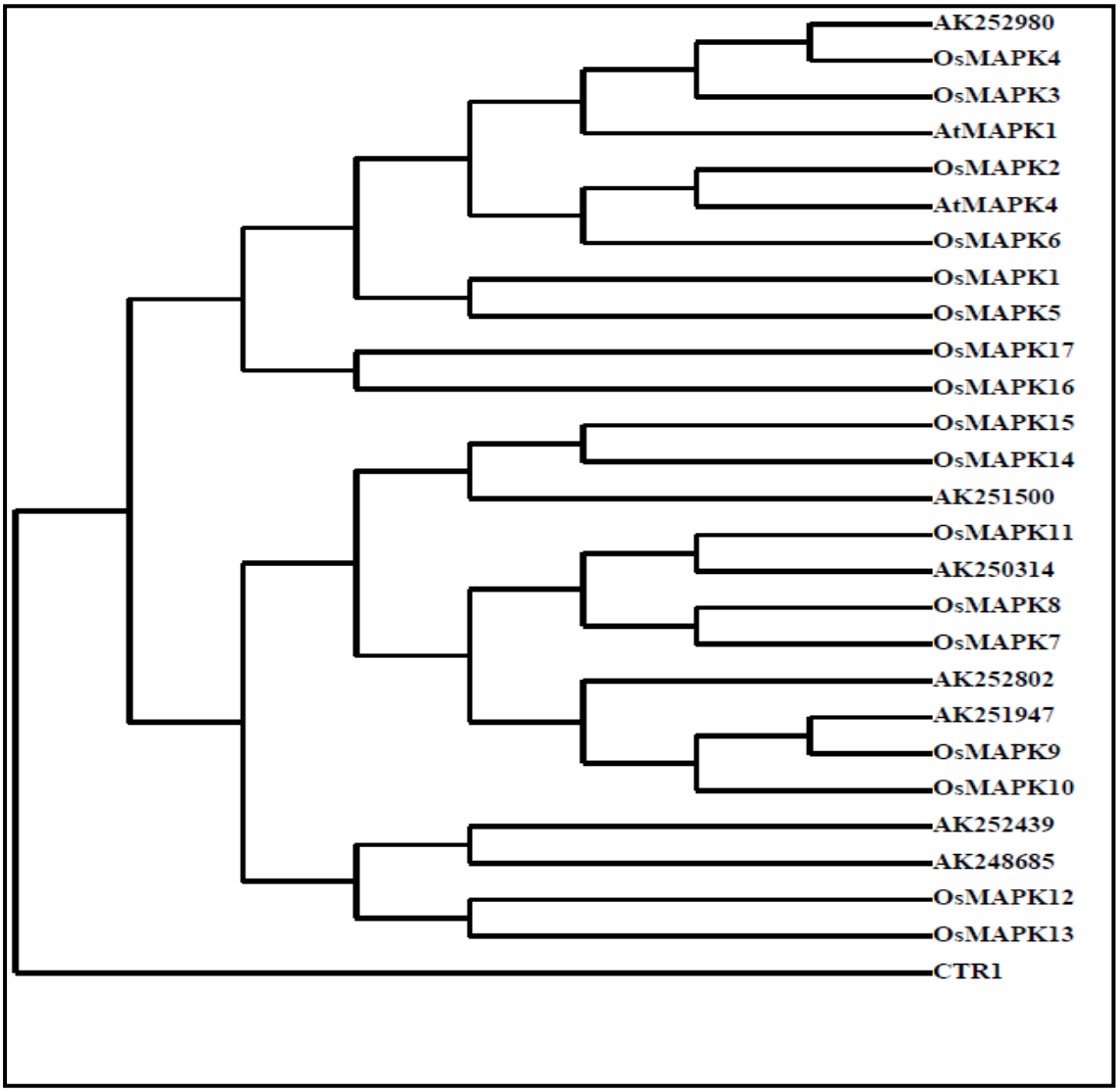


Figure 3.1 Phylogenetic tree illustrating the relationship of barley clones; the rice mitogen-activated protein kinases (*OsMAPK1-OsMAPK17*), *Arabidopsis* MAPK (*AtMAPK1* and *AtMAPK4*) and CTR1.

HvMAPK4 belongs to the MAPK C group with a TEY activation domain motif, and encodes a protein with 369 amino acids of 42 KDa in size (Fig 3.2).

```
HvMAPK4       MAMMVDPPNGIGNHGKHYYTMWQTMFEIDTKYVPIKPIGRGAYGIVCSSINQETNEKVAI 60
OsMAPK4       MVMMVDPPNGMGNQGKHYYTMWQTLFEIDTKVVPIKPIGRGAYGIVCSSINRATNEKVAI 60

HvMAPK4       KKINNVFDNRVDALRTLRELKLLRHLRHENVIALKDIMMPIHRRSFKDVYLVSELMDTDL 120
OsMAPK4       KKINNVFDNRVDALRTLRELKLLRHLRHENVIALKDIMMPVHRRSFKDVYLVYELMDTDL 120

HvMAPK4       HQIVKSSQPLSNDHCQYFLFQLLRGLKYLHSAGILHRDLKPGNLLVNANCDLKICDFGLA 180
OsMAPK4       HQIIKSSQPLSNDHCQYFLFQLLRGLKYLHSAGILHRDLKPGNLLVNANCDLKICDFGLA 180

HvMAPK4       RTNNTKGQFMTEYVVTRWYRAPELLLCCDNYGTSIDVWSVGCIFAELLGRKPIFPGTECL 240
OsMAPK4       RTNNTKGQFMTEYVVTRWYRAPELLLCCDNYGTSIDVWSVGCIFAELLGRKPIFPGTECL 240

HvMAPK4       NQLKLIVNVLGTMSEADLAFIDNSKARKYIKSLPYTPGIPLSSMYPQAHPLAIDLLQKML 300
OsMAPK4       NQLKLIVNVLGTMSEADIEFIDNPKARKYIKTLPYTPGIPLTSMYPQAHPLAIDLLQKML 300

HvMAPK4       VFDPSKRISVTQALEHPYMSPLYDPSANPPAQVPIDLDIDENIGTDMIREMLWQEMLQYH 360
OsMAPK4       VFDPSKRISVTEALEHPYMSPLYDPSANPPVQVPIDLDIDENLGVDMIREMMWQEMLHYH 360

HvMAPK4       PEAARMVNM 369
OsMAPK4       PEVVAGVNM 369
```

Figure 3.2 Sequence alignment of barley HvMAPK4 and rice OsMAPK4 amino acid.

TEY: MAPK4 tripeptide activation domain motif. All the amino acid differences are highlighted by underlining.

3.2 *HvMAPK4* cloning and transformation

The pBluescript plasmid bearing the full length barley cDNA clone was used as template in a PCR reaction using *Pfu* to generate a PCR fragment with the *XbaI* restriction site at the 5' and 3' ends of the ORF. Both the vector (pTopoActin-OCS) and the PCR product were digested with *Xba*1 to provide sticky ends. The 5810 bp and 1128 bp DNA fragments for the vector and insert, respectively, were purified from an agarose gel (Fig. 3.3). The molar ratio of 1:3 (vector to insert) was followed for the ligation, and then the ligated vector and insert were transformed into competent cells of *E. coli*.

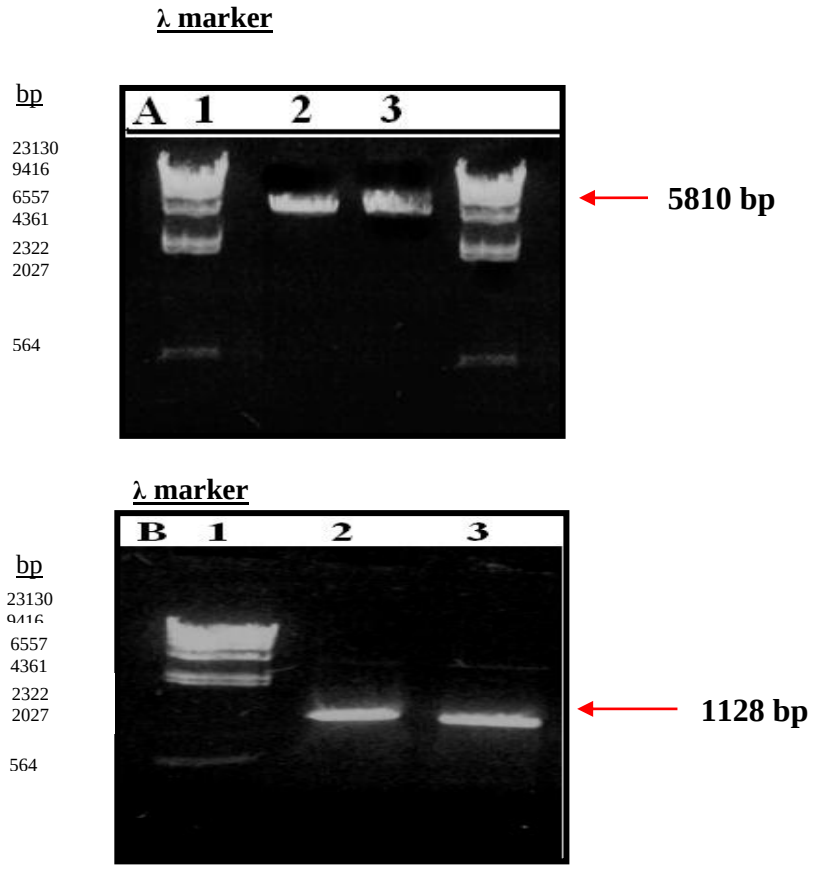


Figure 3.3 Agarose gel electrophoresis of DNA

A. 1 λ *Hind*III marker (see Appendix 5.2). 2 and 3, 5µl of *Xba*I digested and purified plasmid, pTopoActin-OCS.

B. 1 λ Hind III marker, 2 and 3, 5 µl purified DNA of *Xba*I digested HvMAPK4 PCR product (1128bp).

A colony hybridisation was performed to check for the positive colonies of *E. coli* by using both reverse and forward *HvMAPK4* primers to label the *HvMAPK4* gene with digoxigenin in a PCR reaction (Fig.3.4A) and using this as a probe for the hybridisation. At the end of hybridisation, the positive colonies appeared as a purple colour (Figure 3.4B).

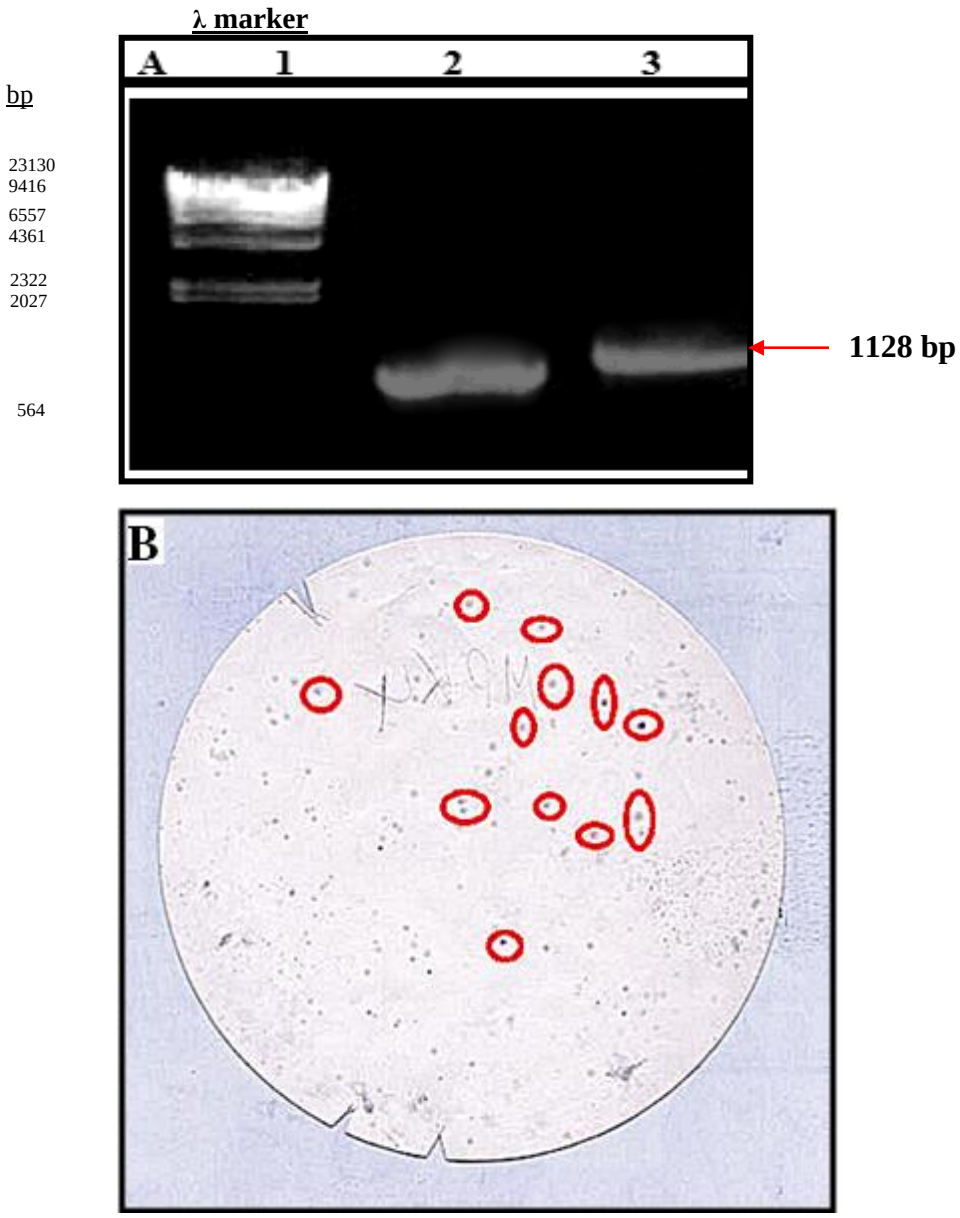


Figure 3.4 Colony hybridisation

A. Agarose gel electrophoresis of *HvMAPK4* DNA probe, 1, λ*Hind*III marker, 2, 5 µl of amplification of *HvMAPK4* 3, 5 µl of amplification of *HvMAPK4*, labelled with digoxigenin showing a shift in size.

B. Colony hybridization showed the positive colonies contained Actin- HvMAPK4-OCS in the transformed cells of *E. coli*. Circled colonies were picked for analysis.

Ten positive colonies were selected to verify the orientation of the HvMAPK4 gene by using the restriction enzyme *Hind*III; eight colonies were found to have the antisense orientation, while just two colonies were found to have overexpression orientation (Fig. 3.5). One colony from both orientations was chosen for further cloning.

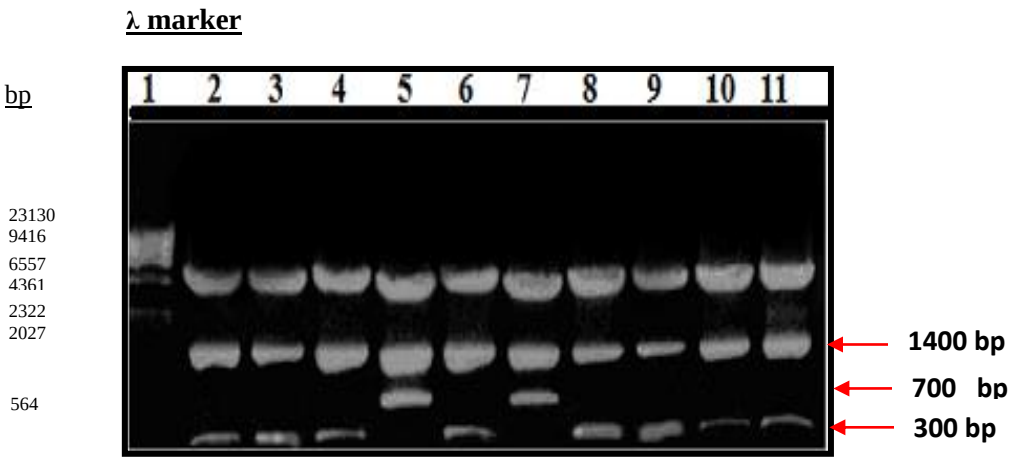


Figure 3.5 Agarose gel electrophoresis of the pActin-HvMAPK4-OCS construct extracted from transformed cells of *E. coli*, 1 λ *Hind*III marker, plasmid DNA digested with *Hind*III restriction enzyme to check the orientation of the insert: bands 2, 3, 4, 6,8,9,10,11 are in the antisense orientation {1.4, 0.3 and 5.1 kbp}; bands 5 and 7 are in the overexpression orientation {1.4, 0.7 and 4.7kbp}.

Plasmid DNA was digested with *Apa*I restriction enzyme to cut out the *Actin* promoter-*HvMAPK4-OCS* terminator fragment (2700 bp Fig. 3.6A), and this insert was purified and ligated into the vector pBWBVec.8 which was previously digested with the *Apa*I restriction enzyme and transformed into competent cells of *E. coli*. Colonies were selected and a PCR reaction was carried out using 1-2 µl of bacterial solution as template in a PCR reaction with the *HvMAPK4* primers to check for positive colonies (Figure 3.6B). Two positive colonies (one colony for each orientation) were selected and the plasmid DNAs were purified and transformed to *A. tumefaciens*.

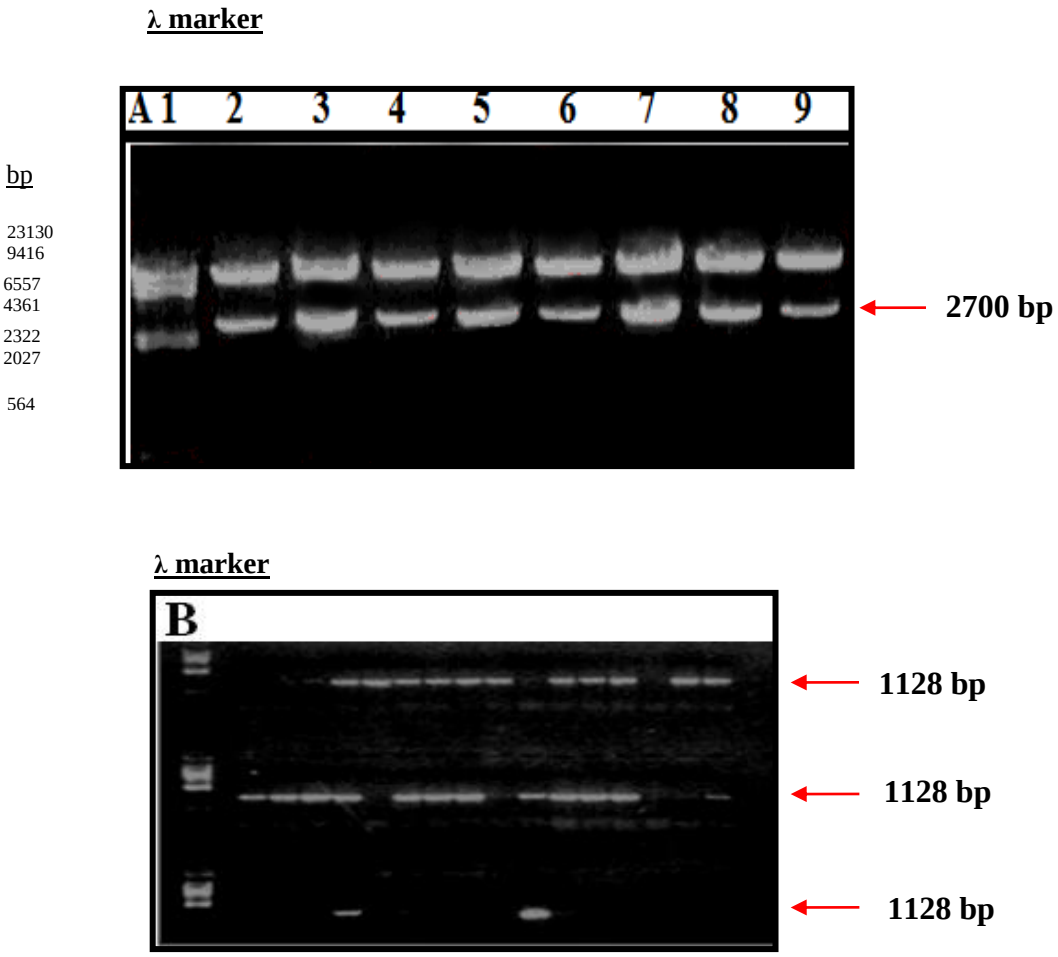


Figure 3.6 Agarose gel electrophoresis

A. Lane 1, λ *Hind* III marker; lanes 2-9, 5 µl of plasmid DNA for Actin- HvMAPK4-OCS in vector pWBvec.8 digested with *Apa*1

B. PCR products using putative pWBvec8: Actin-HcMAPK4-OCS plasmid DNA as template with HvMAPK4 forward and reverse primers, positive bands 1128 bp.

A PCR reaction was carried out to check for the positive colonies of *A. tumefaciens* (Fig. 3.7A), and a digestion with the *Hind*III restriction enzyme for plasmid extracted from *Agrobacterium* bacteria was performed to confirm the orientation of the *HvMAPK4* (Figure 3.7B). The HvMAPK4 open reading frame from the overexpression construct was fully sequenced in order to rule out any introduced mutations. Finally one overexpression and one antisense construct in *A. tumefaciens* were selected to start the transformation into immature embryos of barley (Fig. 3.8 for T-DNA structure).

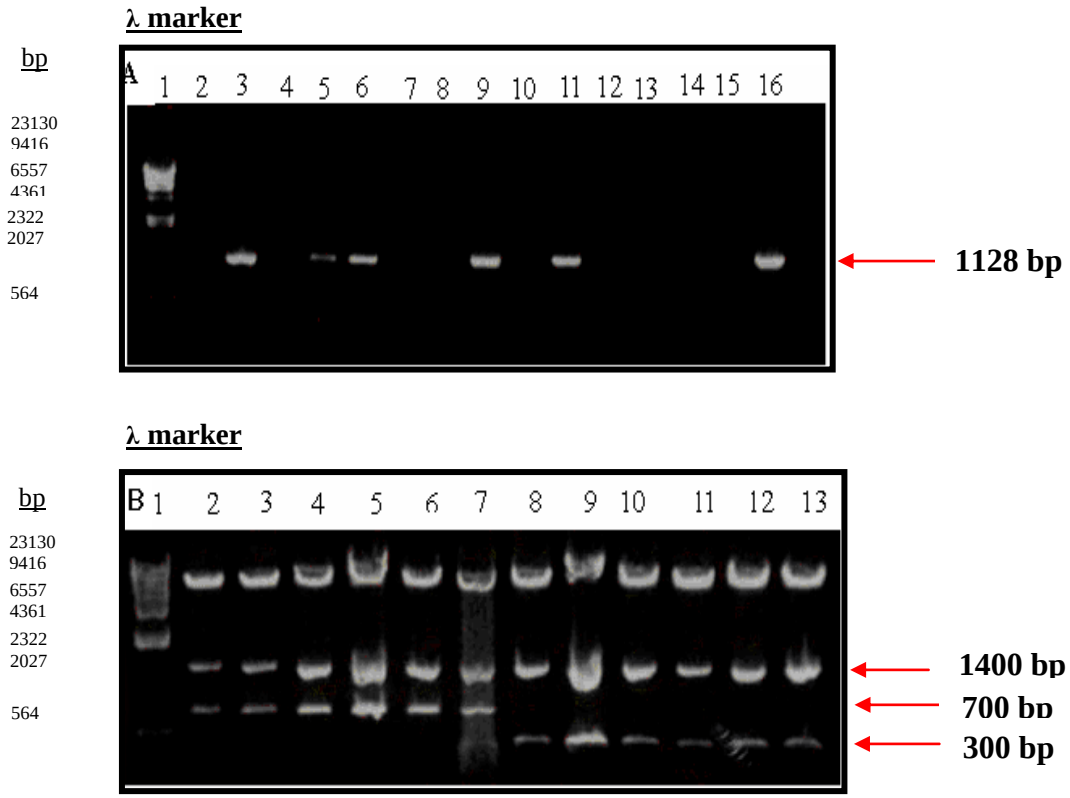


Figure 3.7 Agarose gel electrophoresis

A. PCR products of the *HvMAPK4* gene in the binary vector pWBVec.8: Actin-HvMAPK4-OCS extracted from *Agrobacterium*. Positive bands (3, 5, 6 overexpression constructs with forward primer of *HvMAPK4* and reverse primer of OCS, 9 and 11 antisense constructs with reverse primer of *HvMAPK4* and reverse primer of OCS, 15 and 16 negative and positive control, respectively).

B. Digested plasmid DNA extracted from *Agrobacterium* with the *Hind*III restriction enzyme to check the orientation: bands 2-7, overexpression orientation {1.4, 0.7 and 4.7kbp}; bands 8-13 are antisense orientation {1.4, 0.3 and 5.1 kbp}.

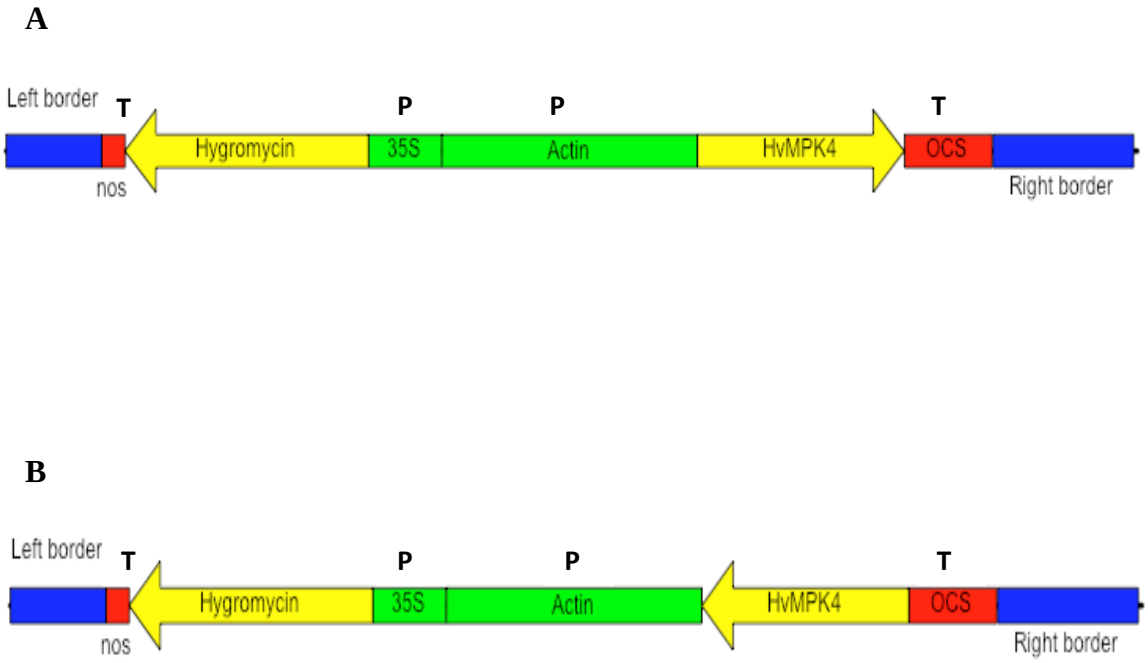


Fig. 3.8 Structure of the T-DNA constructs used for barley transformation.

P: Promoter, T: Terminator

A. *HvMAPK4*-overexpression construct.

B. *HvMAPK4*-antisense construct.

3.3 Barley transformation

Agrobacterium-tumefaciens mediated barley transformation was performed using immature embryos of *Hordeum vulgare* cultivar Golden Promise. The *Agrobacterium* strain EHA105 was used with binary vector pWBVec.8 containing the hygromycin gene as a selectable marker for callus and plant selection during the course of tissue culture.

A total of 200 immature embryos of 1.5-2.5 mm diameter were used for *Agrobacterium* transformation, a total of 19 transgenic plants were obtained, the transformation efficiencies varied from 6 to 13.3% in different experiments, and the total average of transformation efficiency was 9.5% (Table 3.1). The procedure of Tingay *et al*. (1997) and Bartlett *et al.* (2007) were followed with some modifications during the transformation and barley tissue culture. Different stages of tissue culture were followed after the infection of immature embryos, including callus selection stage; regeneration stage and rooting medium are shown in Fig. 3.9.

3.4 Analysis of transgenic barley plants

The second generation of transgenic barley lines (including *HvMAPK4*-antisense, *HvMAPK4*-overexpression and empty binary vector) were analysed by PCR. All the seeds for different barley backgrounds were germinated and selected on hygromycin (100 mg/l). The genomic DNA was prepared from the leaves and checked firstly for quality with tubulin reverse and forward primers (Fig. 3.10A, 3.11A and 3.12A). Secondly, different PCR reactions were performed to check for presence of the correct transgenes, the results of the PCR reactions with *HvMAPK4* reverse primer and *OCS* reverse primer revealed the presence of *HvMAPK4* in antisense direction for antisense transgenic plants (Fig. 3.10C), while the PCR reactions with *HvMAPK4* forward primer and *OCS* reverse primer revealed the presence of *HvMAPK4* in the sense direction for overexpression transgenic plants (Fig. 3.11C). Primers for the *hygromycin* gene were used in PCR reactions to reveal the presence of this gene in all of the transgenic plants; the results for the *hygromycin* gene analysis are shown in Fig. 3.10B, 3.11B and 3.12B. All of the PCR products for *tubulin*, *HvMAPK4* and *hygromycin* genes showed the expected sizes, which were 217, 1128 and 1068 bp, for each gene, respectively. All of the transgenic plants for each construct appeared superficially phenotypically normal and did not show any obvious abnormalities in their growth and fertility.

Table 3.1 Summary of results of different experiments of *Agrobacterium*-mediated barley transformation.

`

Experiment	*HvMAPK4* gene orientation	No. of infected IE	No. of Hygromycin resistant callus	No. of Regenerated plant	No. of +ve PCR plant	% transformation efficiency
1	Antisense	75	21	11	10	13.3
2	Overexpression	50	10	5	3	6
3	Empty vector (pWBVec.8)	75	12	8	6	8
Total		200	46	24	19	9.5

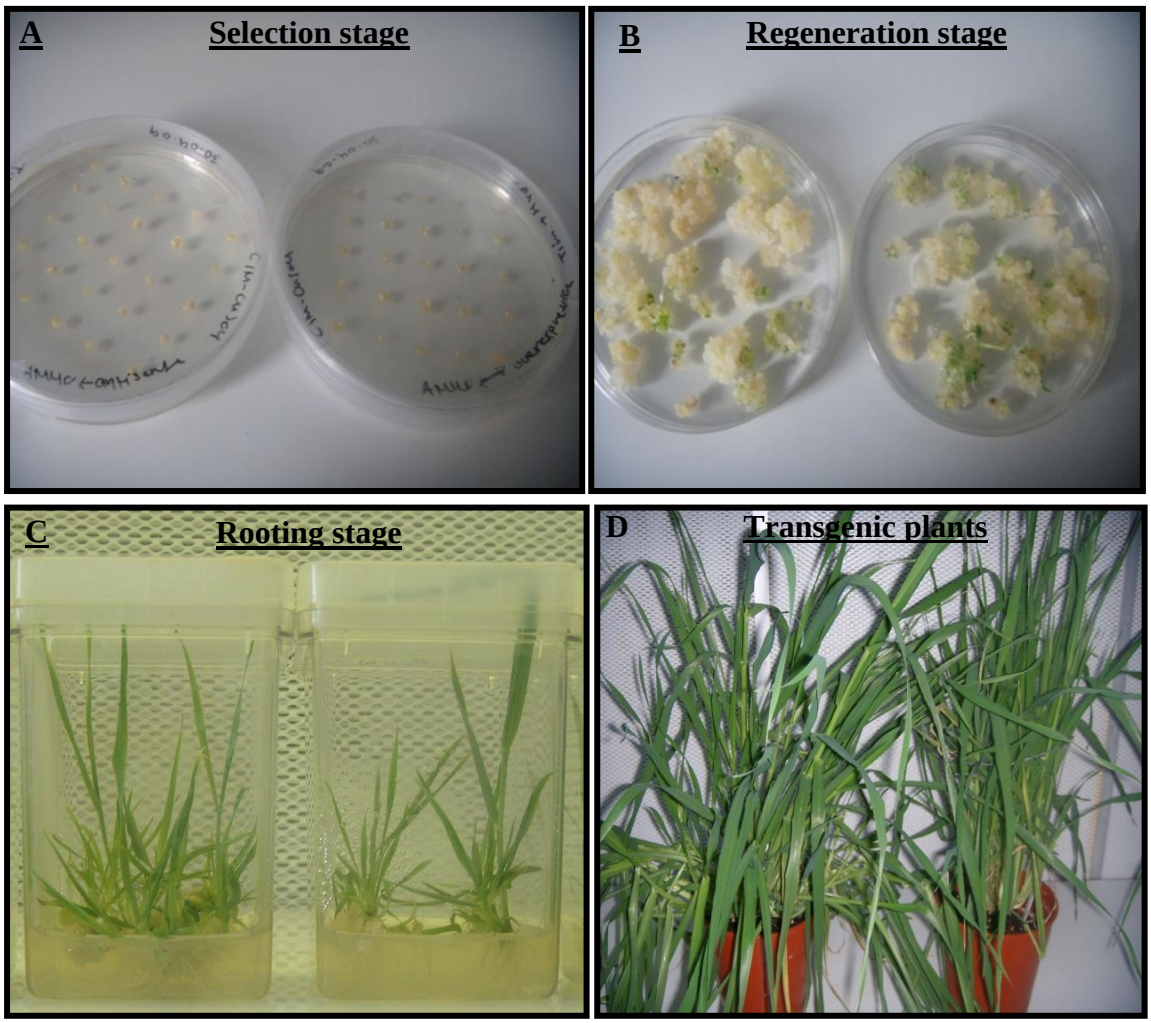


Figure 3.9 Stages of barley transformation

A. Transformed IEs of barley on selection medium (25 embryos in each CIM plate)

B. Green patches on regeneration medium for both overexpression and antisense constructs.

C. Plantlets on rooting medium for both overexpression and antisense construct.

D. Transgenic barley (overexpression and antisense construct).

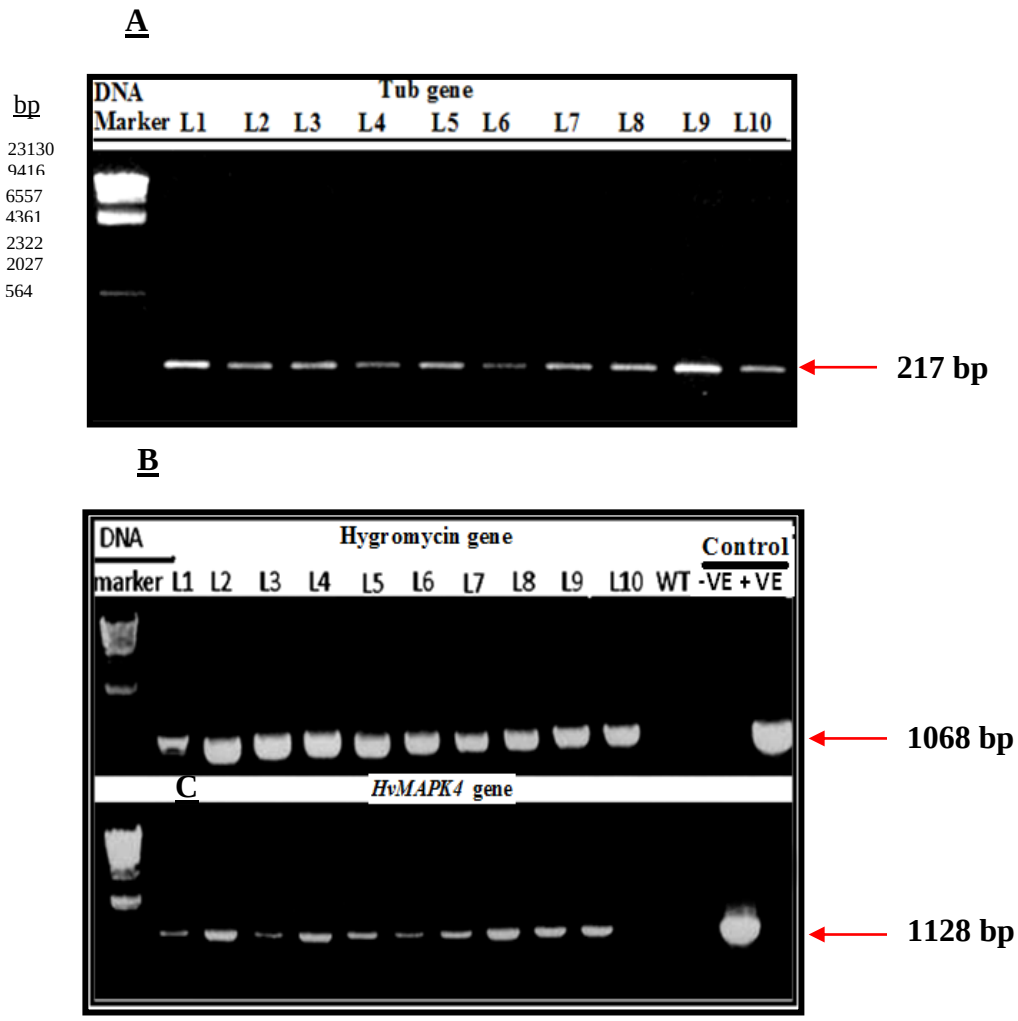


Figure 3.10 PCR products from DNA of 2nd generation transgenic plants containing the *HvMAPK4*-antisense **construct.**

A. PCR products with *tubulin* primers.

B. PCR products with *hygromycin* primers.

C. PCR products with *HvMAPK4* reverse primer and *OCS* reverse primer.

DNA marker is λ *Hind*III marker; L1-L10 represent different independent transgenic of *HvMAPK4*-antisense lines.

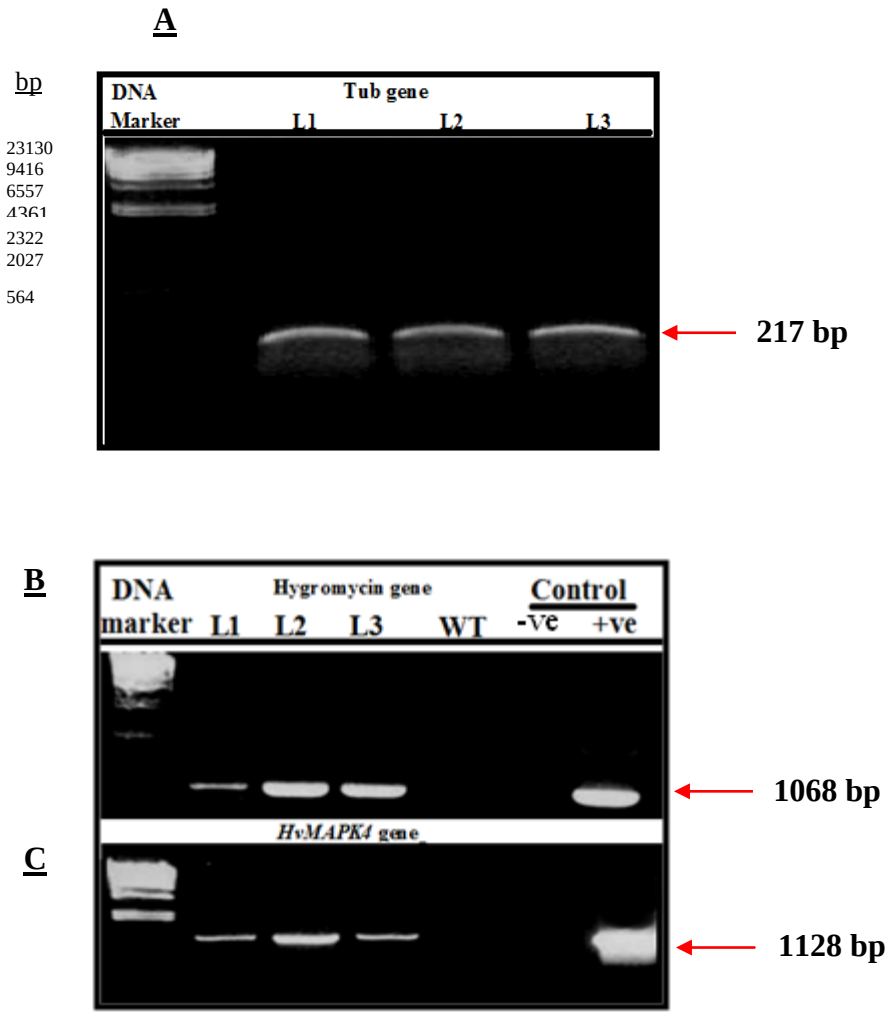


Figure 3.11 PCR products of 2nd generation of transgenic plants with *HvMAPK4*-overexpression construct.

A. PCR products with *tubulin* primers.

B. PCR products with *hygromycin* primers.

C. PCR products with *HvMAPK4* forward primer and *OCS* reverse primer.

DNA marker is λ *Hind* III marker. L1, L2 and L3 represent three independent transgenic of overexpression HvMAPK4 lines

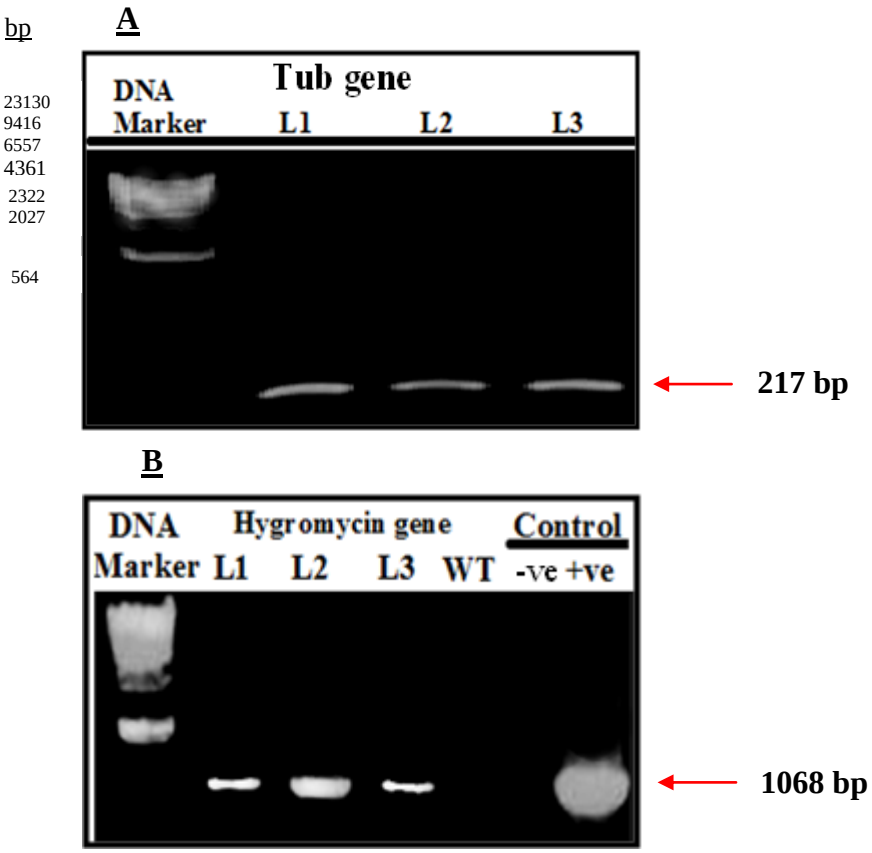


Figure 3.12 PCR products of 2nd generation of transgenic plants with empty binary vector (pWBVec.8) construct.

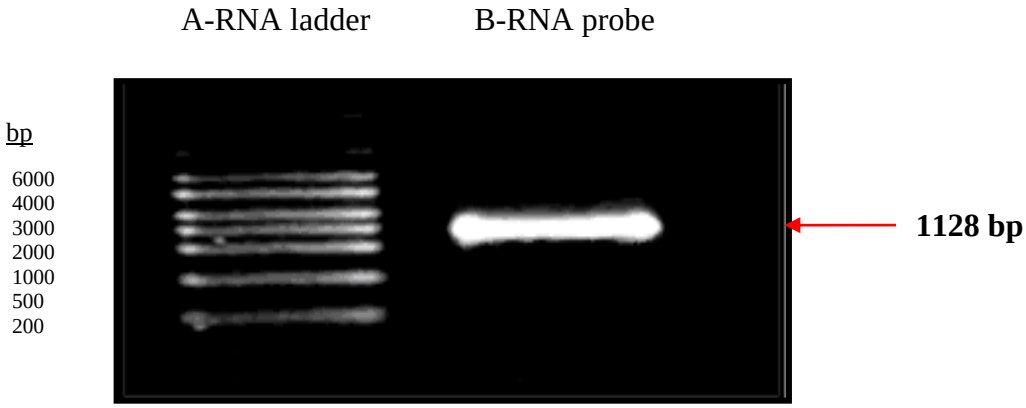
A. PCR products with *tubulin* primers.

B. PCR products with *hygromycin* primers.

DNA marker is λ *Hind*III marker. L1, L2 and L3 represent three independent transgenic lines.

3.5 RNA blot analysis of *HvMAPK4* expression in transgenic lines

The gene expression of *HvMAPK4* was analysed in the second generation of *HvMAPK4*-overexpression and *HvMAPK4*-antisense plant lines along with wildtype plants. All transgenic seeds were germinated and selected on hygromycin (100 mg/l), then the plants were grown on under normal conditions. Three independent transgenic lines from *HvMAPK4*-antisense were selected because these lines showed a good growth patterns and produced much more seeds than other lines. 10 µg of total RNA from barley leaves (three different lines for each construct) was separated on a denaturing agarose gel (Fig. 3.14B, 3.15B, for *HvMAPK4*-overexpression and *HvMAPK4*-antisense lines, respectively). The fractionated RNA was blotted onto nylon membranes and hybridised with an antisense single-stranded *HvMAPK4* RNA probe (Fig. 3.13).



3.13 Antisense RNA probe of *HvMAPK4*.

A. High range RNA ladder (see Appendix 5.3).

B. *HvMAPK4* RNA probe (1128 bp).

The results of the RNA blot analysis indicated that the transgenic lines of *HvMAPK4*-overexpression construct showed an enhanced signal for *HvMAPK4* gene expression in all three transgenic lines compared to the base level of *HvMAPK4* expression in the wildtype (Fig. 3.14A), while the results of RNA blot analysis of *HvMAPK4*-antisense transgenic lines showed an obvious suppression of endogenous *HvMAPK4* gene expression in all three lines (Fig. 3.15A). In particular the expression of the *HvMAPK4* gene was suppressed almost completely in transgenic lines 2 and 3 of *HvMAPK4*-antisense plants, with a weak signal still visible in line 1 as compared to the base level of *HvMAPK4* gene expression in wildtype.

Based on these northern blot results, transgenic lines 1, 2 and 3 for *HvMAPK4*-overexpression and 1, 2 and 3 for *HvMAPK4*-antisense constructs were selected for further experiments.

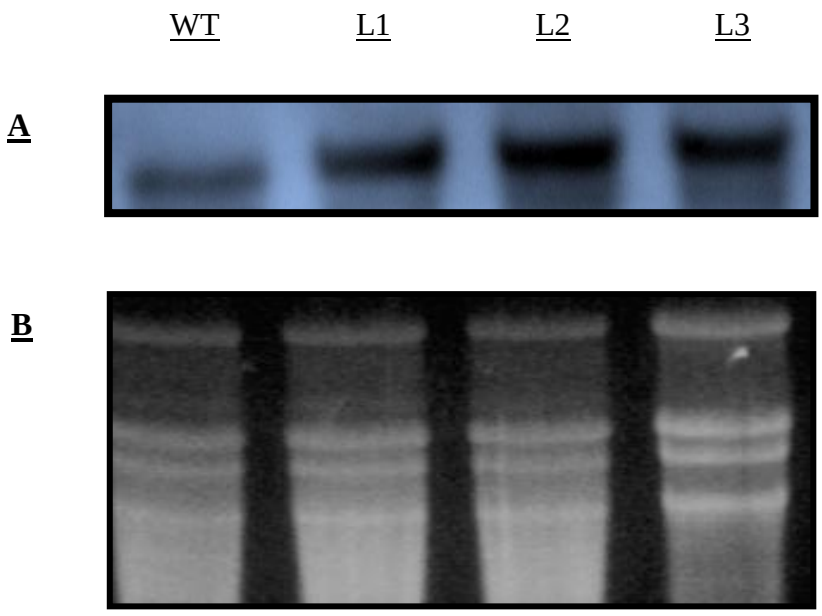


Figure 3.14 Detection of *HvMAPK4* expression in transgenic barley leaves (*HvMAPK4*-overexpression plants) by northern blotting.

A. The expression of the *HvMAPK4* gene in three different independent transgenic lines: L1, L2 and L3. WT represents wildtype

B. 10 µg of total RNA in each lane stained with ethidium bromide and separated on denaturing agarose gel.

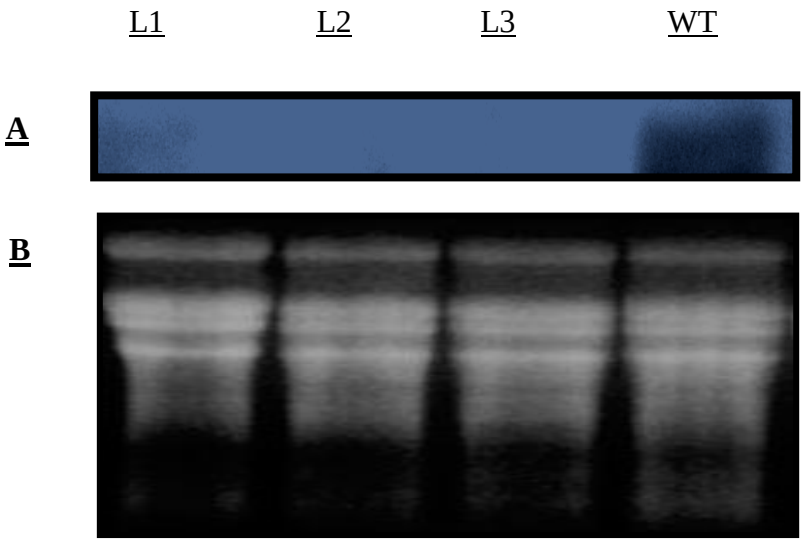


Figure 3.15 Detection of *HvMAPK4* expression in transgenic barley leaves (*HvMAPK4*-antisense plants) by northern blot.

A. The expression profile of *HvMAPK4* gene in three different independent transgenic lines: L1, L2 and L3. WT represents wildtype

B. 10 µg of total RNA in each lane stained with ethidium bromide and separated on denaturing agarose gel.

3.6 Infection assay

3.6.1 The pathogenecity of *M. oryzae* on barley cv. Golden Promise

The wildtype strain Guy-11 of the pathogenic fungus *M. oryzae* (see Fig. 3.16A) was used to inoculate 7 days old barley plants (cv. Golden Promise), to establish suitable conditions for infection. The inoculation process was carried out inside a plastic cover to provide high level of humidity, which is a crucial factor to fungal penetration. The disease symptoms were evident on the youngest leaves five days post-inoculation.

After 7 days of inoculation, the lesion number and size were measured, the lesion number was 6.34 lesions per leaf, and the size was 4.30 mm per leaf, disease severity was 4.3 according to the description of Valent *et al.* (1991).

The lesions varied from round to oval spots on barley leaves, but all of these spots showed a gray centre surrounded by yellow margins (Fig. 3.16B), the pathogenic fungus was reisolated from infected leaves.

All of these results confirmed the strong virulent effect of the pathogen *M. oryzae* on barley plants, and the same inoculation conditions were used to challenge the transgenic plants.

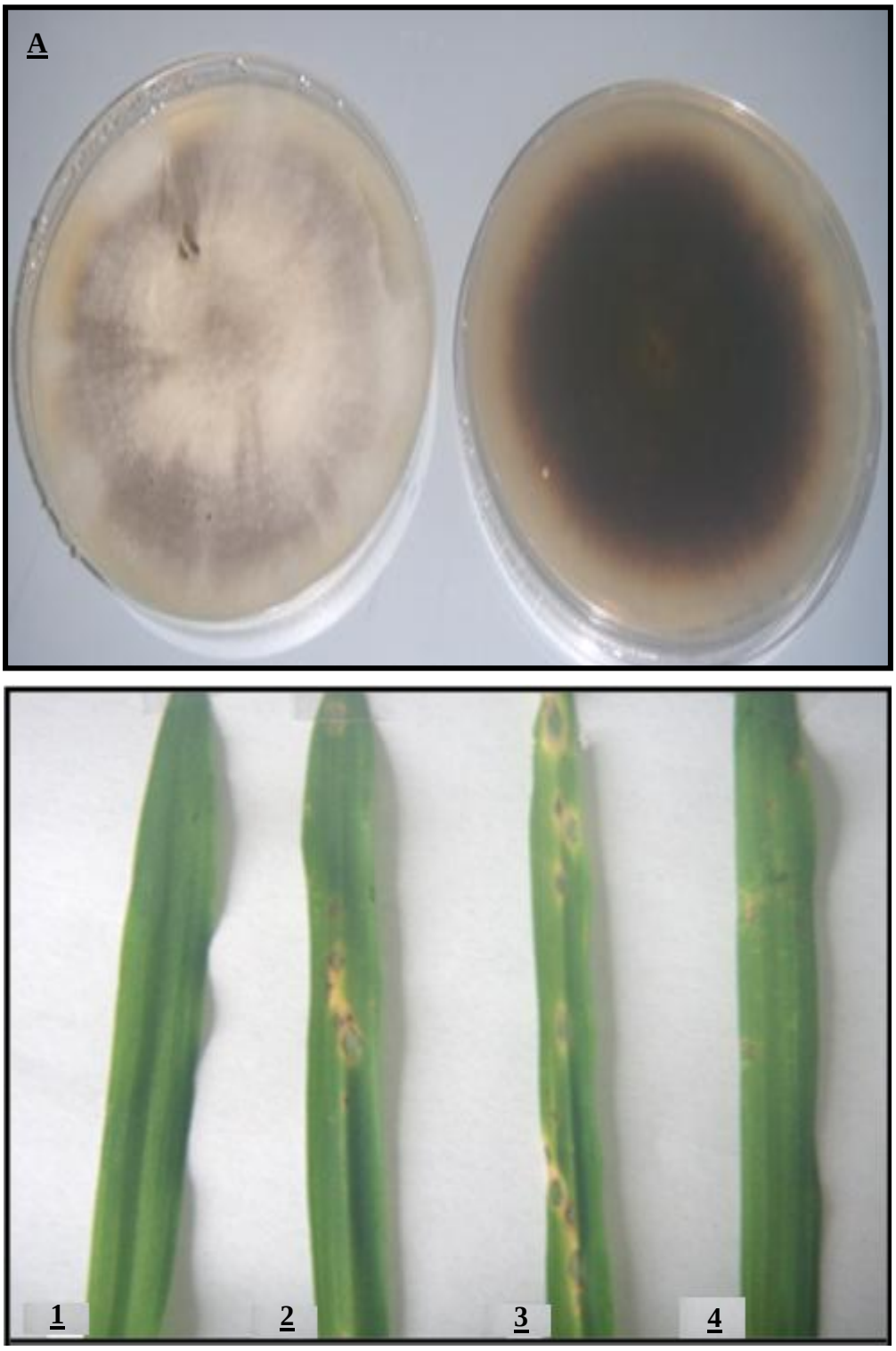


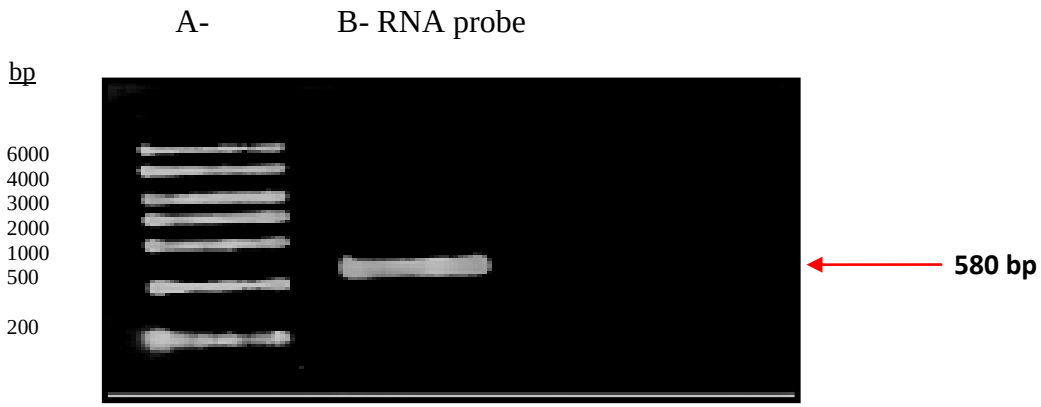
Figure 3.16 Barley infection assay.

A. 2 week old *in vitro* growth of *M. oryzae* (strain Guy-11) on CM plates

B. Disease symptoms on barley leaves, 1 non-inoculated leaf, 2, 3 and 4 inoculated leaves.

3.6.2 Expression profile of *HvMAPK4* and *HvPR1* genes in barley leaves after challenging with *M. oryzae*

The expression of *HvMAPK4* and *HvPR1* genes was investigated in barley wildtype (Golden Promise) leaves in response to the infection with *M. oryzae*. A total of 10 µg of RNA were extracted from barley leaves at 0, 24 and 48 h post-inoculation, and separated on a denaturing gel (Fig. 3.18B, 3.19B for *HvMAPK4* and *HvPR1*, respectively), blotted onto a nylon membrane and probed with antisense single-stranded RNA probe (Fig. 3.17 for antisense RNA probe of *HvPR1*).



3.17 Antisense RNA probe of *HvPR1*.

A. High range RNA ladder (see Appendix 5.3)

B. *HvPR1*RNA probe (580 bp)

The analysis of *HvMAPK4* gene expression showed that this gene was expressed pre and post-inoculation with the pathogen, but the level of expression was up-regulated at 24 and 48 post-inoculation compared to the base level of endogenous *HvMAPK4* gene expression at time 0 (Fig. 3.18A).

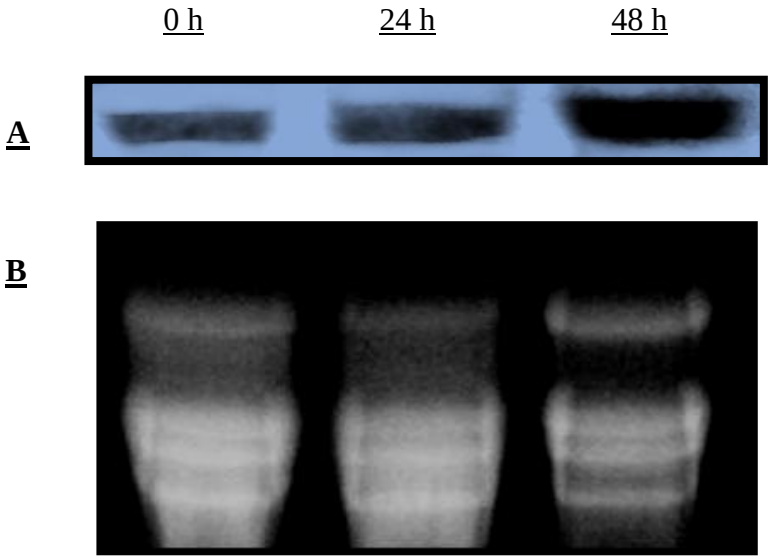


Figure 3.18 Detection of *HvMAPK4* expression in barley leaves by northern blotting in response to *M. oryzae.*

A. The expression profile of *HMAPK4* at 0, 24 and 48 hours post-inoculation.

B. 10 µg of total RNA in each lane stained with ethidium bromide and separated on denaturing agarose gel.

No expression was seen for the *HvPR1* gene at time 0, but the level of expression was found to be enhanced in response to the infection at 24 and 48 h post-inoculation (Fig 3.19A). Thus both the *HvMAPK4* and *HvPR1* genes were shown to be inducible in barley leaves as a response to the challenge with *M. oryzae*.

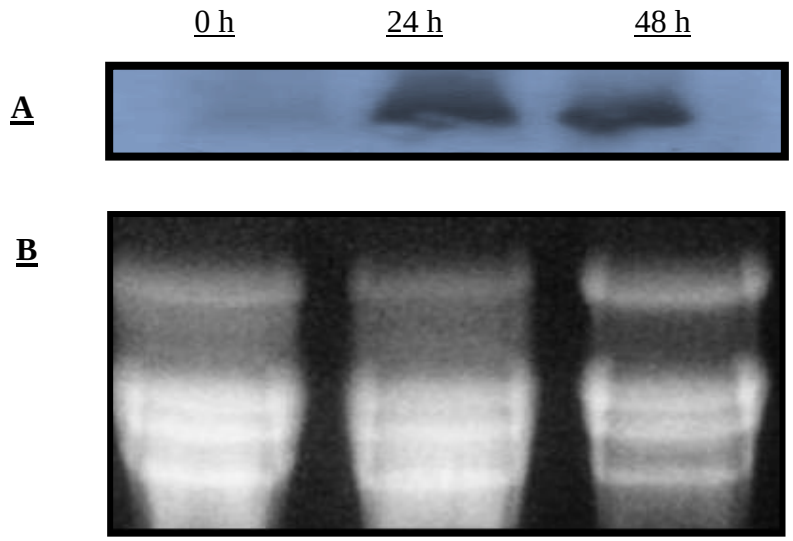


Figure 3.19 Detection of *HvPR1* expression in barley leaves by northern blotting in response to *M. oryzae*.

A. The expression profile of *HvPR1* at 0, 24 and 48 hours post-inoculation.

B. 10 µg of total RNA in each lane stained with ethidium bromide and separated on denaturing agarose gel.

3.7 Phytohormone measurements

To determine the response of barley plants (wildtype, *HvMAPK4*-overexpression and *HvMAPK4*-antisense plants) to the challenge with *M. oryzae*, the alteration in the levels of endogenous phytohormones including salicylic acid, jasmonic acid and ethylene were measured in systemic (uninfected) leaves pre and post-inoculation with the pathogen at 0, 24 and 48 h.

3.7.1 Salicylic acid levels

Salicylic acid levels were measured in different barley backgrounds because of its importance in systemic acquired resistance. The results showed that the wildtype plants responded by increasing endogenous salicylic acid level ten fold within 48 h of infection with *M. oryzae*. The level of salicylic acid after infection was found to be 5240 ng/g fresh weight of barley leaves compared to the basal level which was 530 ng/g. Salicylic acid levels were found to be much higher in the *HvMAPK4*- antisense plants in contrast with the *HvMAPK4*-overexpression plants and wildtype (Fig. 3.20). The amounts of salicylic acid in *HvMAPK4*-antisense plants were significantly different ($p < 0.05$ using ANOVA) at 24 and 48 h post-inoculation than the amounts in the wildtype and *HvMAPK4*-overexpression plants, while there was no significant difference between the wildtype and *HvMAPK4*-overexpression plants in their salicylic acid levels at all of the time points. The highest accumulation of salicylic acid at 8130 ng/g was found in *HvMAPK4*-antisense plants at 48 h post-inoculation. This data suggests that at least basal levels of HvMAPK4 can repress the accumulation of SA in barley after infection, but enhanced HvMAPK4 levels do not further reduce SA.

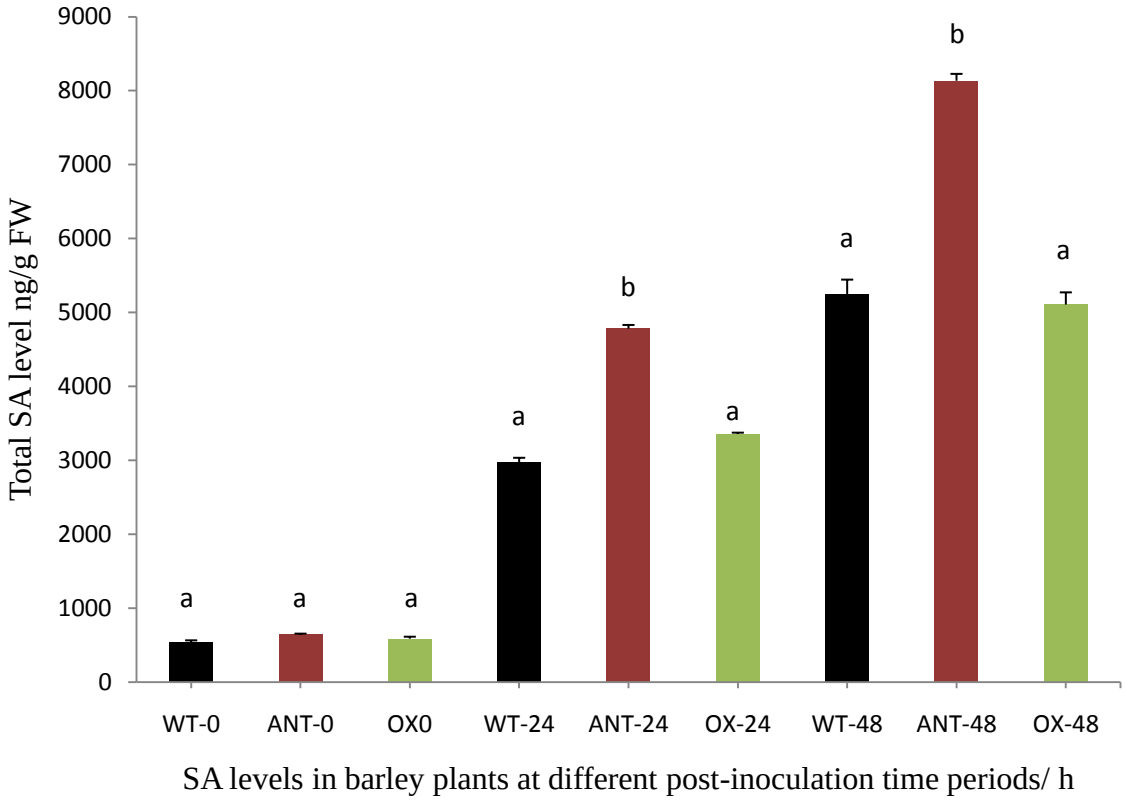
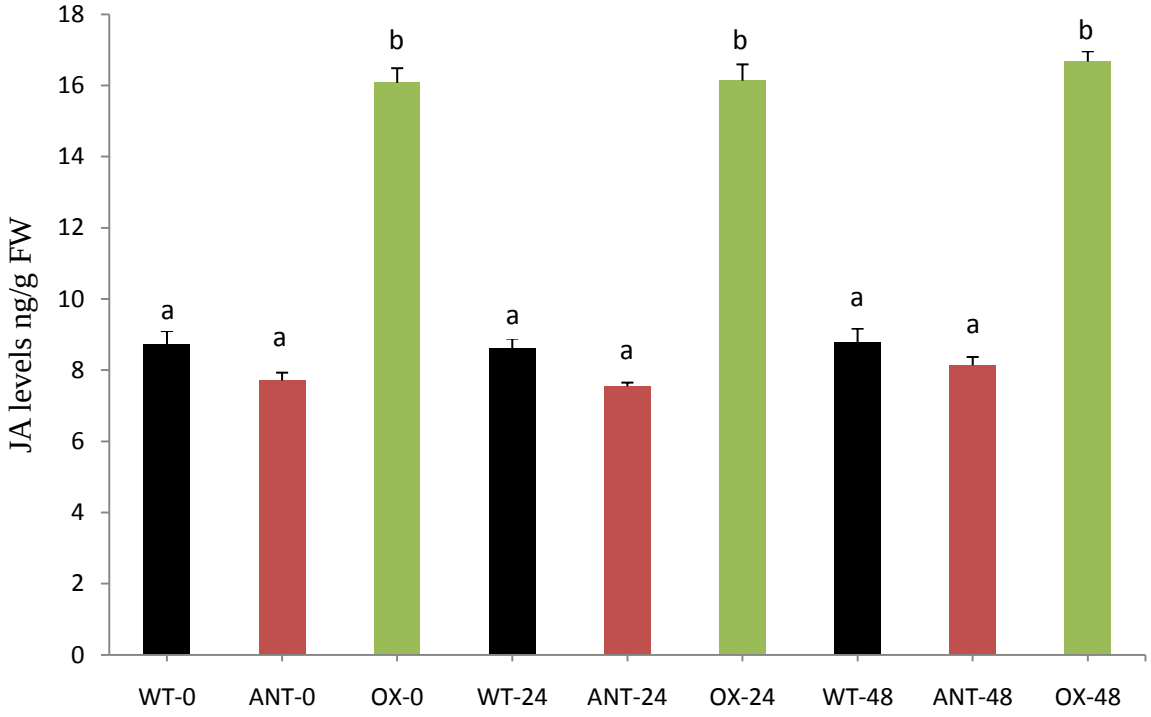


Figure 3.20

Endogenous level of salicylic acid in barley leaves at 0, 24 and 48 hours post-inoculation with *M. oryzae*. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference ($P<0.05$) at each time period using Tukey's test. WT: wildtype, ANT: *HvMAPK4*-antisense plant, OX: *HvMAPK4*-overexpression plant.

3.7.2 Jasmonic acid level

The endogenous levels of jasmonic acid were found not to change during the time course of infection with *M.oryzae* for each barley genotype. However, the level of jasmonic acid was much higher in *HvMAPK4*-overexpression plants and was significantly different ($p < 0.05$) than the levels in wildtype and *HvMAPK4*-antisense plants (Fig. 3.21). Thus, in contrast to the findings on SA levels, *HvMAPK4* appears to act as a positive regulator of JA accumulation, even in the absence of biotic stress.



JA levels in barley plants at different post-inoculation time periods/ h

Figure 3.21

Endogenous level of jasmonic acid in barley leaves at 0, 24 and 48 hours post-inoculation with *M. oryzae*. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference ($P<0.05$) at each time period. WT: wildtype, ANT: *HvMAPK4*-antisense plant, OX: *HvMAPK4*-overexpression plant.

3.7.3 Ethylene level

As shown in Fig. 3.22, wildtype plants responded to the challenge with *M. oryzae* by increasing ethylene production up to 2.25 fold at 48 h post-inoculation, and similar increases in ethylene production were found in the *HvMAPK4*-antisense lines. However, the production of ethylene was much higher in *HvMAPK4*-overexpression plants at all time points with a significant difference compared with the amounts in *HvMAPK4*-antisense and wildtype plants, which were 1.7 and 1.5 fold lower, respectively. The highest amount of ethylene (2320 nl/g/h) was found in *HvMAPK4*-overexpression plants at 48 h post-inoculation. Thus *HvMAPK4* appears to act as a positive regulator of biotic stress induced ethylene.

To summarize the results of the phytohormone measurements, both the jasmonic acid and ethylene production results revealed that the wildtype and *HvMAPK4*-antisense plants responded in very similar way to the infection with the hemibiotrophic pathogen *M. oryzae* at all time points without any significant differences ($p < 0.05$), but there was an obvious enhancement in the level of jasmonic acid and ethylene production in *HvMAPK4*-overexpression transgenic plants. Regarding salicylic acid, the antisense repression of *HvMAPK4* appeared to result in accumulation of more salicylic acid, approximately 1.6 fold than that in wildtype and *HvMAPK4*-overexpression plants, but overexpression of *HvMAPK4* had no effect on SA levels.

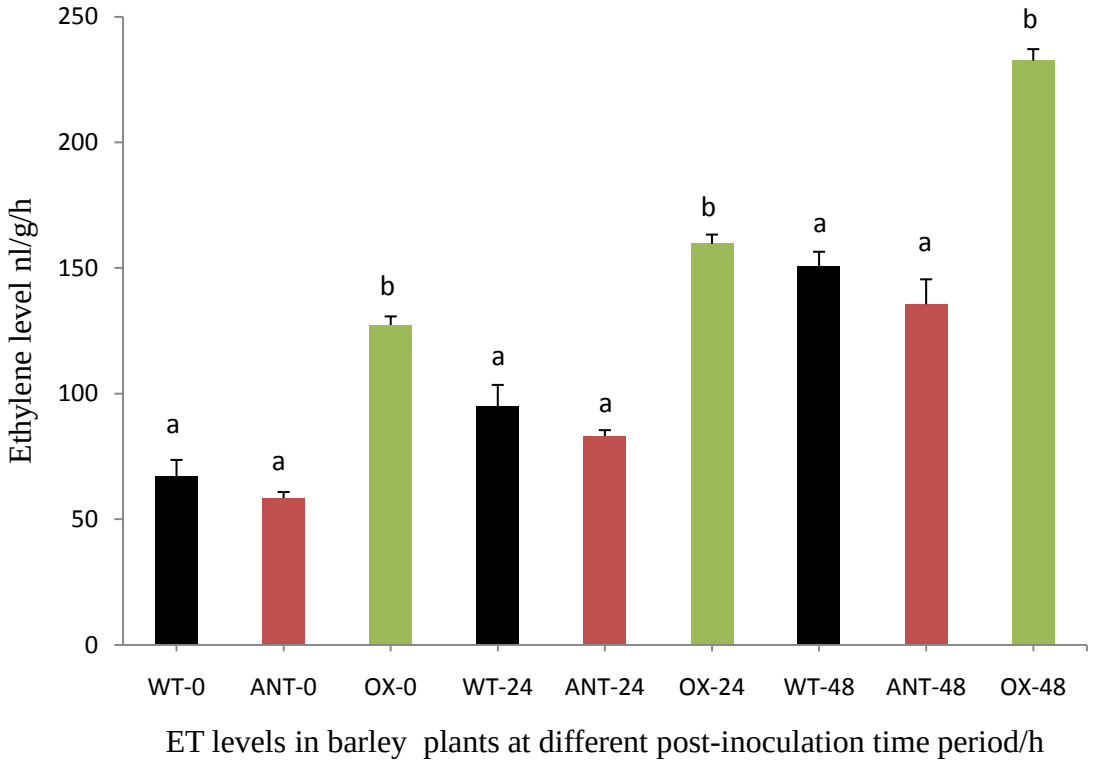


Figure 3.22

Ethylene production by barley leaves at 0, 24 and 48 hours post-inoculation with *M. oryzae*. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference ($P<0.05$) at each time period. WT: wildtype, ANT: *HvMAPK4*-antisense plant, OX: *HvMAPK4*-overexpression plant.

3.8 Hydrogen peroxide level and catalase activity in transgenic and wildtype plants after challenging with *M. oryzae*

Hydrogen peroxide is one kind of ROS in plant with significant importance in several physiological processes and responses to biotic and abiotic stresses.

The measurement of hydrogen peroxide showed significant differences between different barley backgrounds (Fig. 3.23). The *HvMAPK4*-antisense lines showed an increase the level of hydrogen peroxide as a response to the infection with the pathogen, the level of increase was 1.5 fold that of the wildtype at 48 h post-inoculation. There was no significant difference between *HvMAPK4*-antisense and wildtype plants at time 0 and 24 h post-inoculation. The accumulation of hydrogen peroxide was reduced significantly in *HvMAPK4*-overexpression plants, the reduced level was found to be 1.5 fold lower than that in wildtype. The highest amount of hydrogen peroxide (1850 nmol/g) was found in *HvMAPK4*-antisense plant responding to the infection at 48 h post-inoculation, followed by the wildtype.

The results for the catalase activity measurements proved that the *HvMAPK4*-overexpression plants showed the highest activity for catalase at each time point compared to what was measured in wildtype and *HvMAPK4*-antisense plants. The catalase activity was 2.7 fold higher in *HvMAPK4*-overexpression in contrast with *HvMAPK4*-antisense at 48 h post-inoculation (Fig. 3.24), and it is noteworthy that the *HvMAPK4*-antisense plants showed the lowest catalase activity at each time point of infection with significant differences than each wildtype and *HvMAPK4*-overexpression.

These results suggest that the modification in barley *HvMAPK4* gene expression leads to an alteration in the response to the hemibiotrophic pathogen *M. oryzae*. The highest level of hydrogen peroxide was observed in *HvMAPK4*-antisense plants and this was accompanied by a reduction in catalase activity. The opposite was found in the *HvMAPK4*-overexpression plants which accumulated a reduced amount of hydrogen peroxide and showed higher catalase activity at the same time.

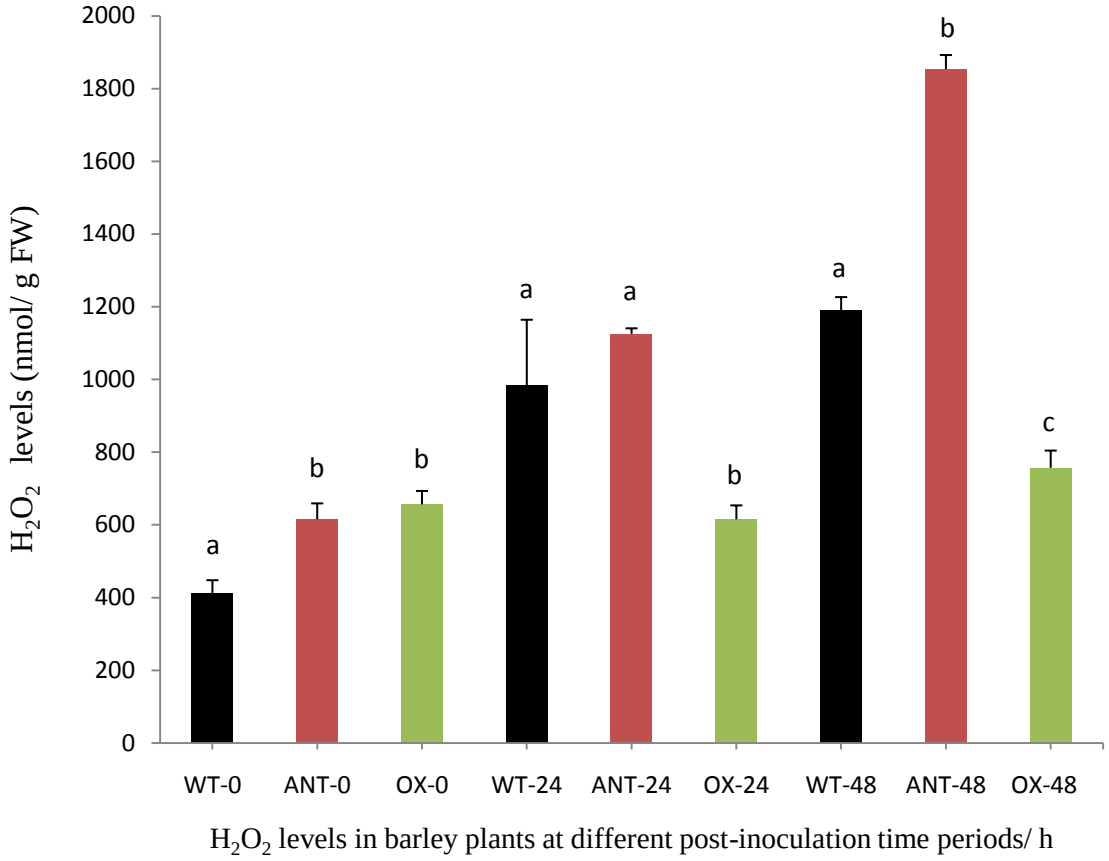


Figure 3.23

Endogenous level of hydrogen peroxide in barley leaves at 0, 24 and 48 hours post-inoculation with *M. oryzae*. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference ($P<0.05$) at each time period. WT: wildtype, ANT: *HvMAPK4*-antisense plant, OX: *HvMAPK4*-overexpression plant.

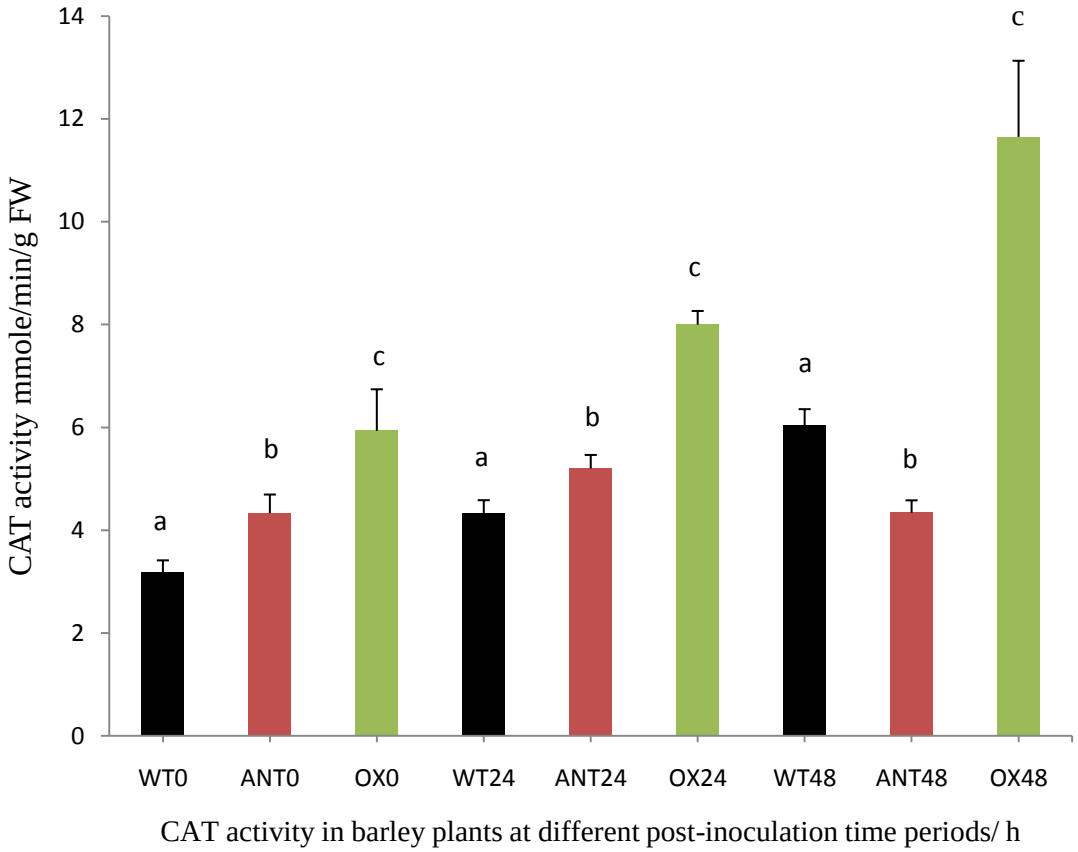


Figure 3.24

Catalase activity in barley leaves at 0, 24 and 48 hours post-inoculation with *M. oryzae*. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference ($P<0.05$) at each time period. WT: wildtype, ANT: *HvMAPK4*-antisense plant, OX: *HvMAPK4*-overexpression plant.

3.9 Disease severity in transgenic and wildtype barley plants

Transgenic and control plants infected with *M. oryzae* were monitored for their disease symptoms. After 7 days of infection, significant differences between barley backgrounds were seen. The *HvMAPK4*-overexpression plants showed a similar sensitive reaction to infection as seen in wildtype plants without any significant difference ($P < 0.05$) in disease parameters. Lesion numbers were 7.5 and 7 lesions/ leaf in *HvMAPK4*-overexpression and wildtype plants, respectively (Fig. 3.25), whilst the disease severity was 4 and 4.6 in both *HvMAPK4*-overexpression and wildtype, respectively.

The antisense suppression of the *HvMAPK*4 gene in barley plants reduced the level of sensitivity to the pathogen compared to the wildtype and *HvMAPK4*-overexpression plants (Fig. 3.26). The lesion number was decreased by up to 57%, whereas the lesion size was reduced by up to 77% compared to that in wildtype plants, and a disease severity of 2.1 was found in *HvMAPK4*-antisense plants with significant differences to what was seen in *HvMAPK4*- overexpression and wildtype plants.

The analysis of *HvPR1* gene expression in the different barley backgrounds showed that this gene was expressed constitutively in the *HvMAPK4*-antisense plants with a strong accumulation at 24 and 48 h post-inoculation as compared to *HvMAPK4*-overexpression plants, which showed a more modest accumulation of *HvPR1* (Fig. 3.27). This contrasts with the pattern of *PR1* gene expression seen in wildtype plant (Fig 3.19) in which no expression was seen prior to infection.

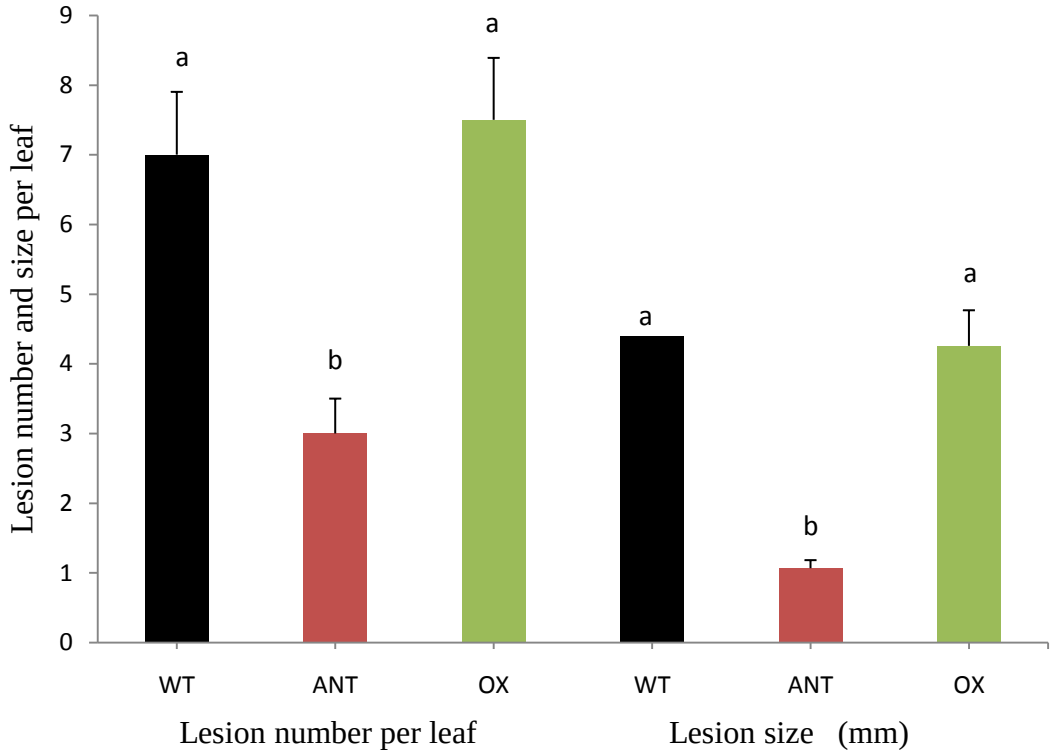


Figure 3.25

Disease parameters of barley wildtype and transgenic plants as a response to *M. oryzae* inoculation. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference at P<0.05. WT: wildtype, ANT: *HvMAPK4*-antisense plant, OX: *HvMAPK4*-overexpression plant.

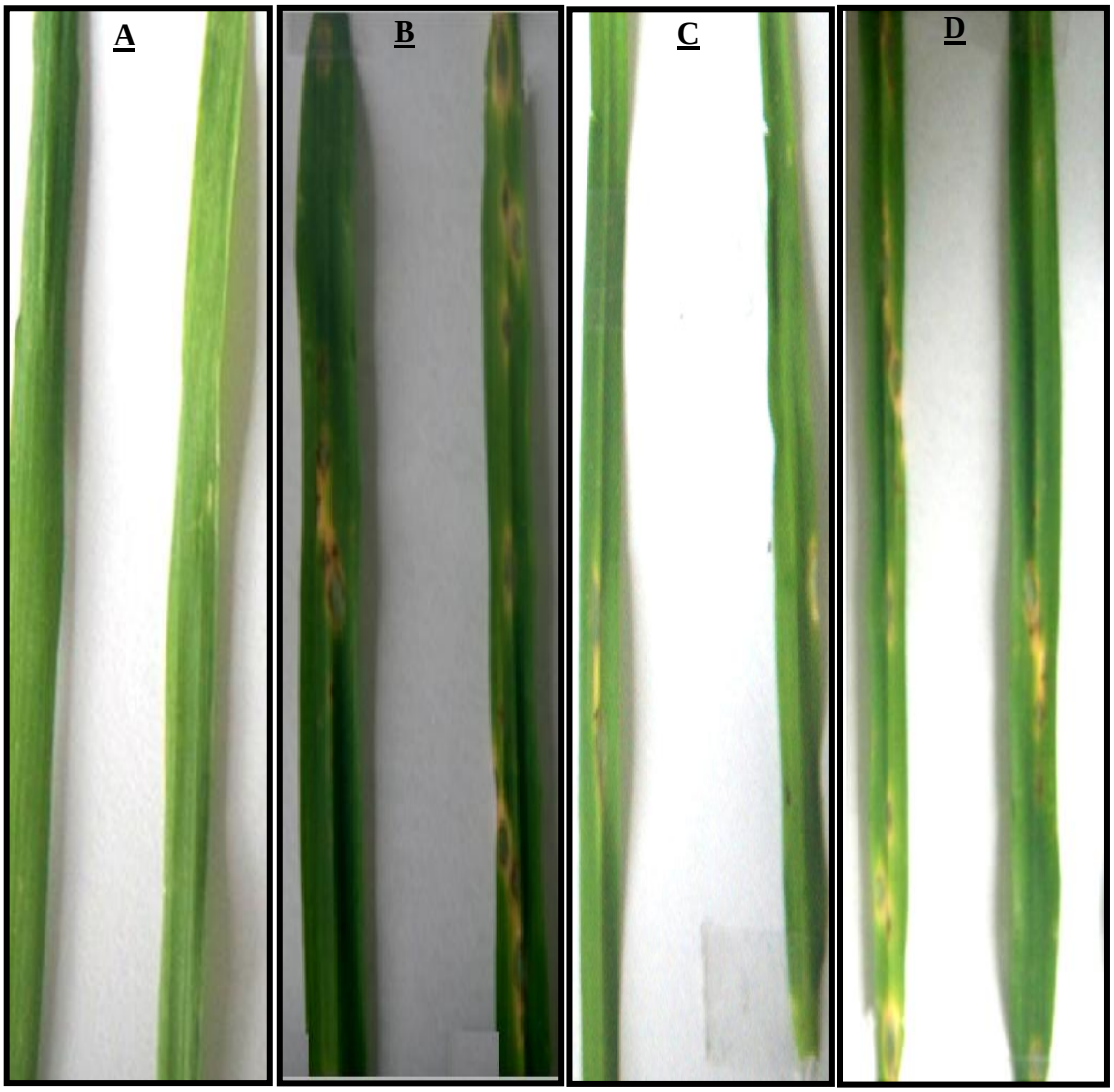


Figure 3.26 Barley leaf segments showing disease symptoms, 7 days post-inoculation.

A. Non-inoculated wildtype plants.

B- Inoculated wildtype plants.

C. Inoculated *HvMAPK4*-antisense plants.

D. Inoculated *HvMAPK4*-overexpression plants.

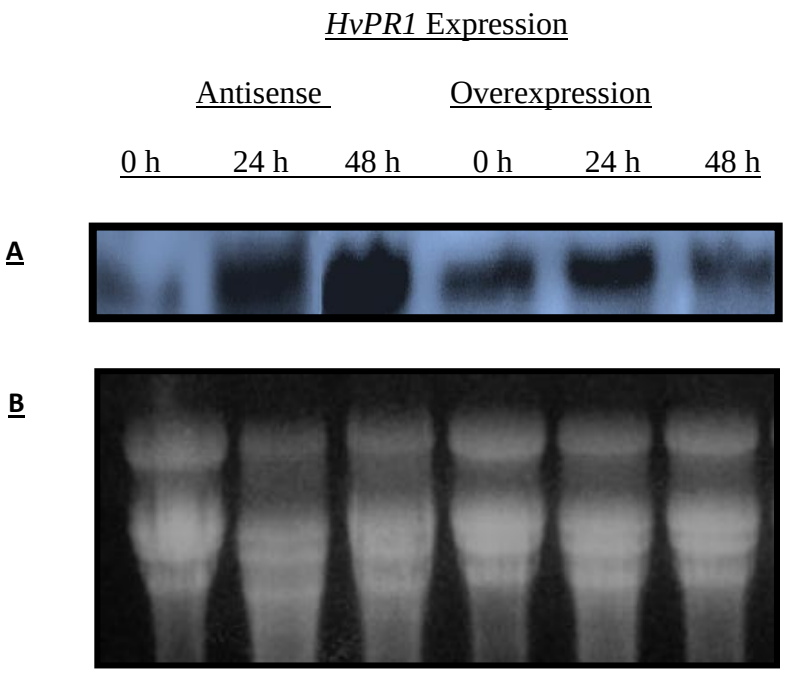


Figure 3.27 Detection of *HvPR1* expression in transgenic barley leaves (antisense and overexpression plants) by northern blot in response to *M. oryzae.*

A. The expression profile of *HvPR1* at 0, 24 and 48 hours post-inoculation.

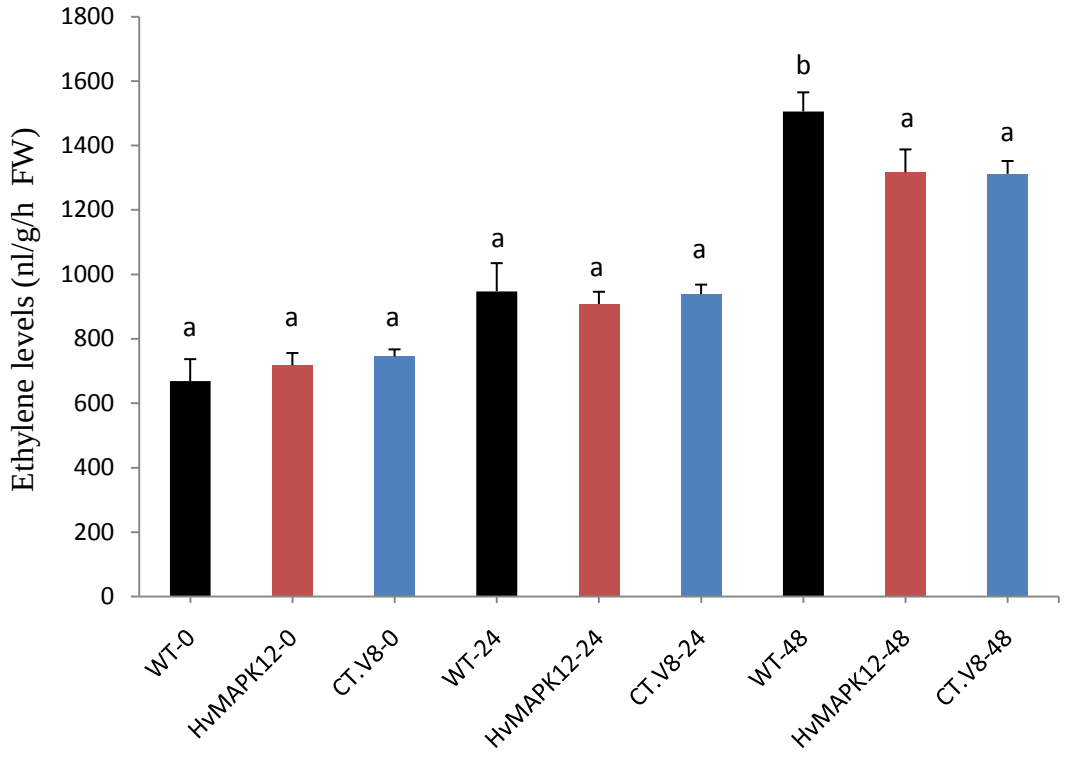
B. 10 µg of total RNA in each lane stained with ethidium bromide and separated on denaturing agarose gel.

3.10 The response of *HvMAPK12*-antisense and empty vector plants to the infection with *M. oryzae.*

The rice MAPK *HvMAPK12* (also known as *OsBWMK1*) has been shown to be implicated in biotic stress and the barley homologue (*HvMAPK12*) has also been studied (Salem Rajab, Heriot-Watt University, personal communication). *HvMAPK12*-antisense plants (courtesy of Salem Rajab) were challenged with hemibiotrophic pathogen *M. oryzae* along with wildtype and transgenic barley bearing the empty vector (pWBVec.8). The results showed that there was no significant difference between these barley backgrounds in their responses to *M. oryzae* infection in terms of ethylene production at 0 and 24 h post-inoculation, whereas at 48 h post-inoculation there was a slight reduction in the production of ethylene in both *HvMAPK12*-antisense and empty vector plants compared to wildtype which showed the highest production of ethylene (1505 nl/g/h) (Fig. 3.28).

Measurement of hydrogen peroxide in these plants after infection showed that the highest level of hydrogen peroxide (1400 nmole/g) was found in the *HvMAPK12*-antisense plant*s* at 48h post-inoculation with a significant difference to the levels seen in wildtype and empty vector plants. No significant differences were observed for any of the barley backgrounds at time 0, while the level of hydrogen peroxide was significantly higher in the *HvMAPK12*-antisense plants at 48 h post-inoculation than both wild type and empty vector plants (Fig. 3.29).

The results for lesion number and lesion size were similar in all of these barley backgrounds, no significant differences were observed for any of the barley backgrounds for all disease parameters (Fig. 3.30). Similar results were found with disease severity, the *HvMAPK12*-antisense was sensitive to the infection with blast pathogen with disease severity of 4.2 without any significant difference compared with wildtype and empty vector controls (4.6 and 4.7, respectively).



ET levels in barley plants at different post-inoculation time/ h

Figure 3.28

Ethylene production by barley leaves at 0, 24 and 48 hours post-inoculation with *M. oryzae*. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference ($P<0.05$) at each time period. WT: wildtype, *HvMAPK12*: *HvMAPK12*-antisense, CT.V8: transgenic plants with empty vector

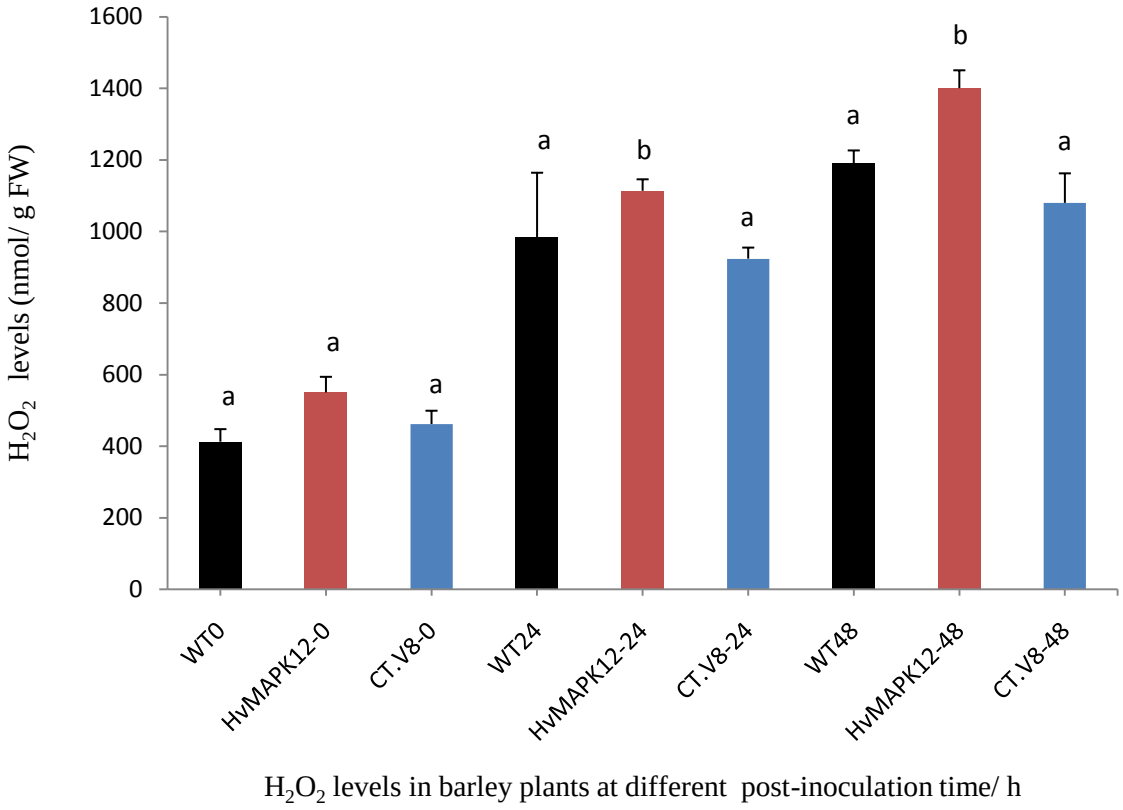


Figure 3.29

Endogenous level of hydrogen peroxide in barley leaves at 0, 24 and 48 hours post-inoculation with *M. oryzae*. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. Different letters show significant difference ($P<0.05$) at each time period. WT: wildtype, *HvMAPK12*: *HvMAPK12*-antisense, CT.V8: transgenic plants with empty vector

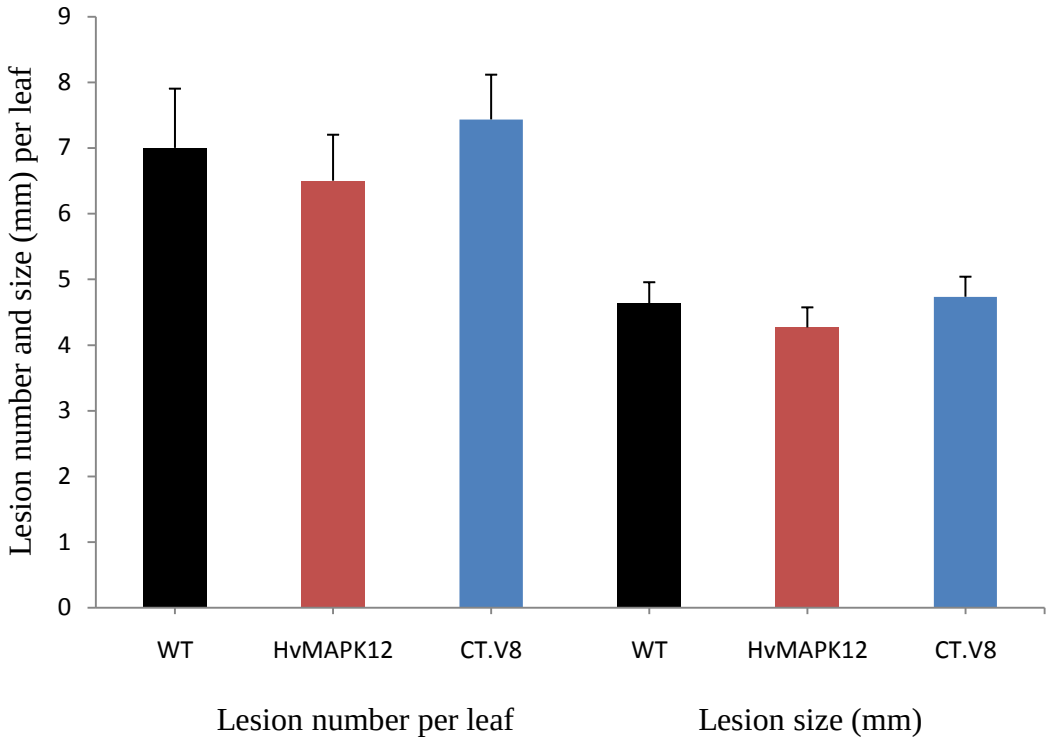


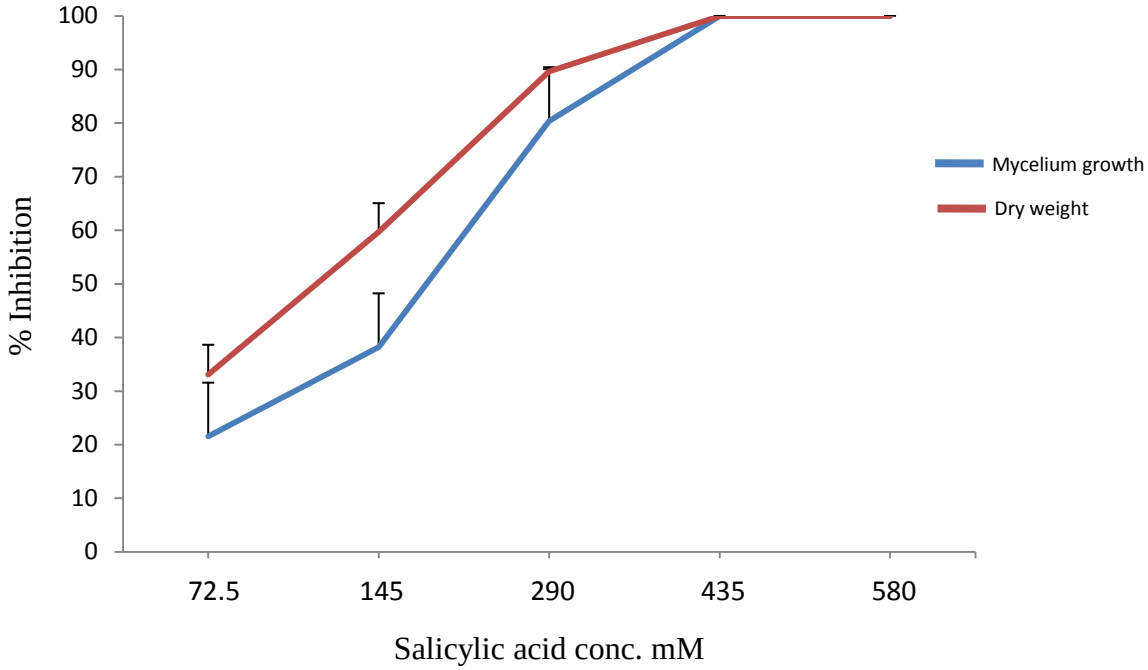
Figure 3.30

Disease parameters of barley wildtype and transgenic plants as a response to *M. oryzae* inoculation. Each data point represents the mean of one measurement each on three lines. Vertical bar indicates standard deviation. WT: wildtype, *HvMAPK12*: *HvMAPK12*-antisense, CT.V8: transgenic plants with empty vector

3.11 The effect of exogenous salicylic acid and hydrogen peroxide on *in vitro* growth of *M. oryzae*.

It was found that the exogenous application of salicylic acid and hydrogen peroxide had a significant influence on both mycelium growth and dry weight of *M. oryzae*. It was clear from the results that the inhibition of fungal growth increased with the increasing chemical concentrations. Salicylic acid at concentrations of 435 and 590 mM were sufficient to inhibit the mycelium growth and dry weight of *M. oryzae* completely, while the percent of inhibition for both mycelium and dry weight were 21 and 33%, respectively, at 72.5 mM of salicylic acid (Fig. 3.31, Fig. 3.32).

The data for hydrogen peroxide indicated the toxic effect of this chemical toward the growth of the pathogen; the concentration of 50 mM was enough to inhibit both mycelium growth and dry weight completely, whereas the concentration of 10 mM was sufficient to inhibit 79 and 89% of mycelium growth and dry weight, respectively (Fig. 3.31, Fig. 3.32).



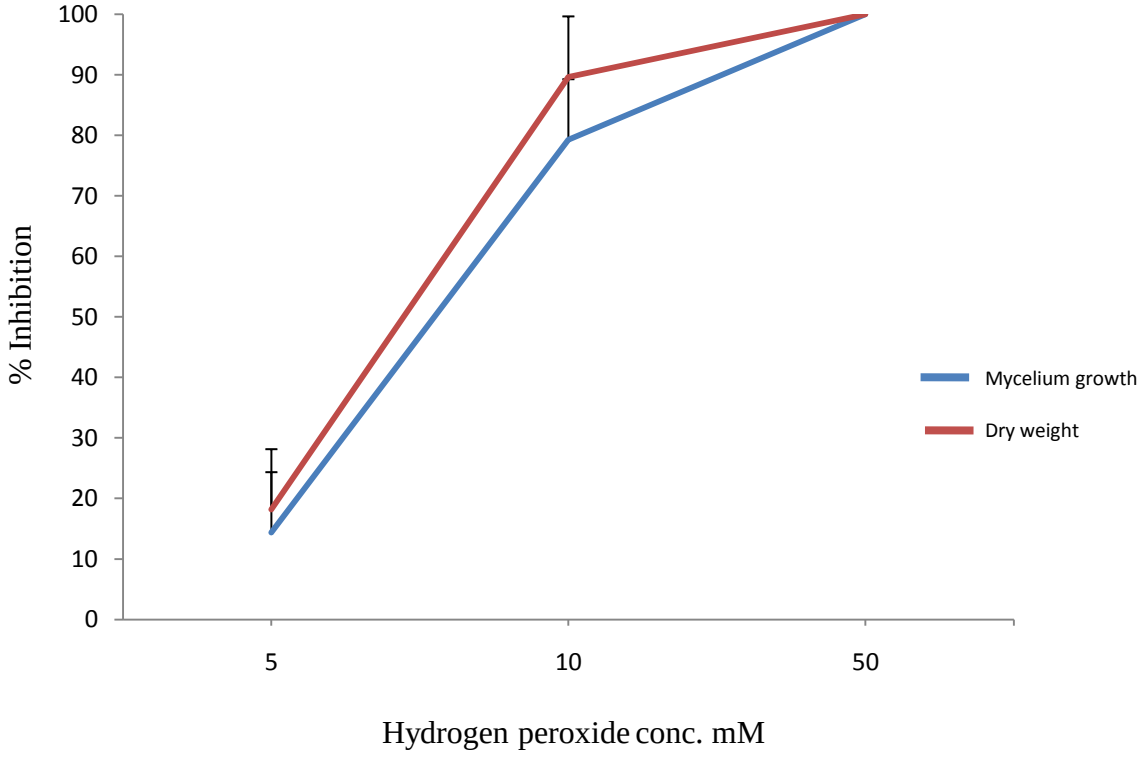


Figure 3.31

The inhibitory effect of exogenous salicylic acid and hydrogen peroxide on *in vitro* growth of *M. oryzae*. Each data point represents the mean of triplicates Vertical bar represents standard deviations of the mean.

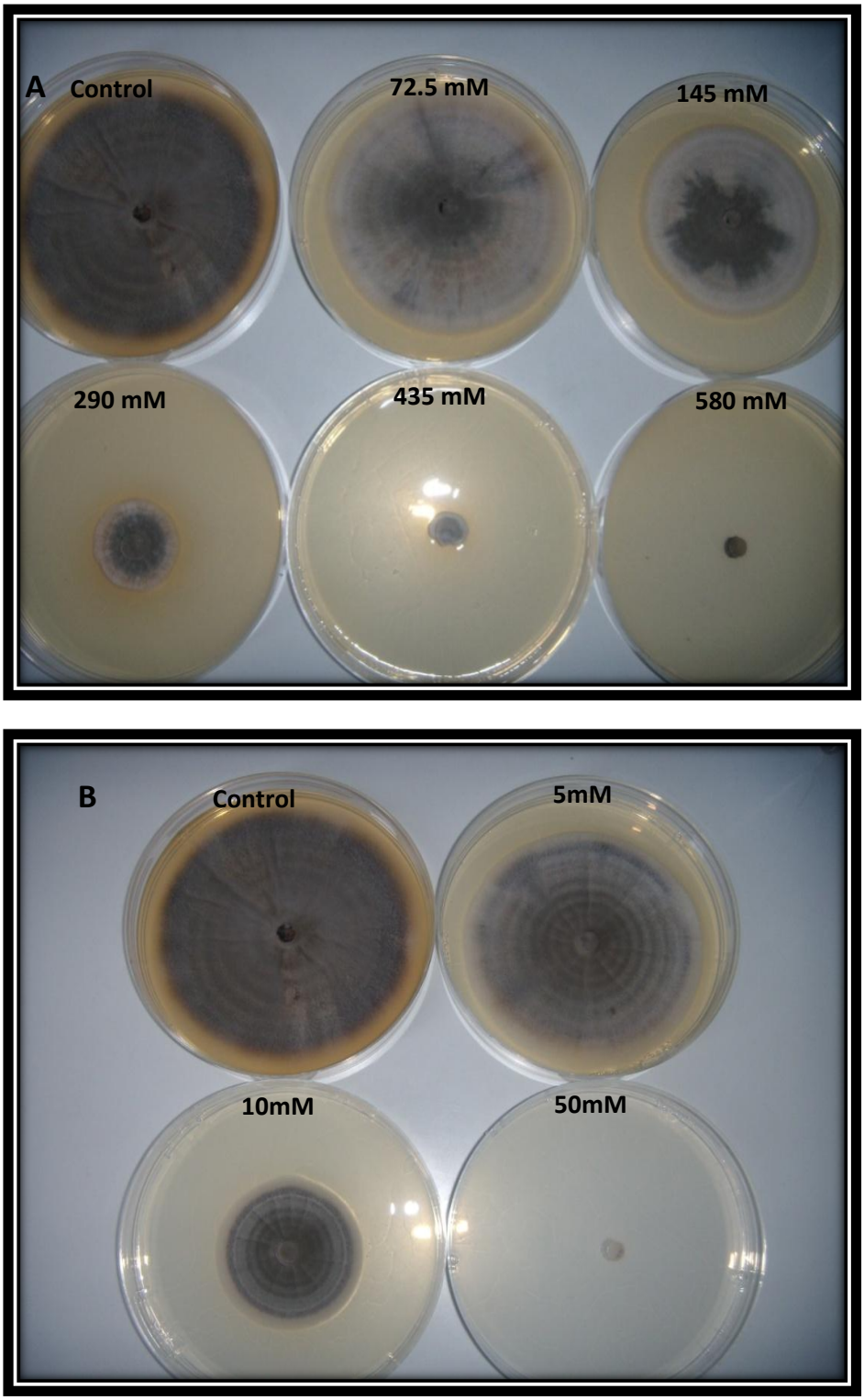


Figure 3.32

The effect of exogenous salicylic acid and hydrogen peroxide on *in vitro* growth of *M. oryzae.*

A. The effect of salicylic acid on mycelial growth of *M. oryzae*.

B. The effect of hydrogen peroxide on mycelial growth of *M. oryzae*.

3.12 Summary of results

The results presented here suggest that the *HvMAPK4* is a negative regulator in barley defence to the hemibiotrophic pathogen *M. oryzae* via the salicylic acid pathway. Evidence for this finding was accumulated from both phytohormone measurements, RNA blot analysis and disease severity.

1- *HvMAPK4*-antisense plants produced more salicylic acid at 48 h post-inoculation as compared to *HvMAPK4*-overexpression and wildtype plants, which accumulated similar amount of salicylic acid.

2- *HvMAPK4*-overexpression plants appeared to accumulate more jasmonic acid and ethylene, whilst there was no significant difference between *HvMAPK4*-antisense and wildtype plants.

3- *HvMAPK4*-antisense plants showed enhancement in its hydrogen peroxide level and reduction in the activity of catalase, but the opposite was found in *HvMAPK4*-overexpression at 48 h post-inoculation.

4-RNA analysis showed that *HvMAPK4*-antisense plants constitutively expressed a molecular marker of SAR (*HvPR1*).

5- *HvMAPK4*-antisense plants showed an enhanced level of resistance to the hemibiotrophic pathogen *M. oryzae*, whilst no significant difference was observed between *HvMAPK4*-overexpression and wildtype plants in terms of disease severity, lesion number and size.

6-*In vitro* experiments revealed the toxic effect of salicylic acid and hydrogen peroxide on the growth of *M. oryzae.* Both mycelium growth and dry weight were inhibited completely at salicylic concentrations 435 and 580 mM, whilst hydrogen peroxide at 50 mM was sufficient to inhibit both mycelium growth and dry weight completely.

CHAPTER 4 DISCUSSION

4.1 Barley *MAPK4*

In recent years tremendous progress has been made toward the understanding of biological functions of plant MAP kinases with many attempts to identify the function and regulation of MAPK gene families in economically important cereal crops.

Different plant MAPKs have been associated with abiotic stresses such as drought and cold (Agrawal *et al.*, 2003a). However, plant MAP kinases have also proved to be involved in defence mechanisms against a wide range of pathogenic fungi and bacteria such as *M. oryzae* and *Xanthomanas oryzae* (Reyna and Yang, 2007; Shen *et al.*, 2010).

The amino acid sequences of seven full length cDNA clones encoding barley MAPK homologues (donated by Dr. K. Sato Okayama University, Japan) were compared with 17 MAPKs known to exist in rice (Reyna and Yang, 2006). The resulting phylogenetic tree showed the relationship of the barley clones and the rice MAPKs, and seven different barley MAPK homologues were identified: *OsMAPK12* (also known as *BWMK1*), a splicing variant of *OsMAPK12*, *MAPK4*, *MAPK9*, *MAPK10*, *MAPK11* and *MAPK14* (Table 4.1).

The alignment of the amino acid sequence deduced from the barley clone with the Genebank accession number AK252980 with the rice amino acid sequence for *OsMAPK4* (also known by other authors as *OsMAPK2* and *OsMSRMK3)* showed a 93.5% similarity. Based on this analysis it is confirmed that AK252980 is the barley homologue of *OsMAPK4* and is named *HvMAPK4* in this study.

Barley *HvMAPK4* was selected for further analysis of its role in the barley response to the hemibiotrophic pathogen *M. oryzae,* because of the results of Reyna and Yang (2006) which showed that the rice *OsMAPK4* gene was up-regulated as a response to the infection with blast pathogen, while the other MAPKs including *OsMAPK9*, *OsMAPK10*, *OsMAPK11* and *OsMAPK14* showed no induction after challenging rice plant with *M. oryzae*. In addition to the involvement of rice *OsMAPK4* in the plant response to the pathogen, several papers showed that this gene is involved in rice responses to environmental stresses as well. The mRNA level of *OsMAPK4* was up-regulated in rice responses to high level of salinity, sugar starvation and cold treatment, salicylic acid and heavy metals, suggesting that this gene functions in both biotic and abiotic stress-signalling pathways (Fu *et al.*, 2002; Agrawal *et al.*, 2003a).

Regarding rice MAPKs, to date three different MAPKs have been investigated to identify their functions in plant defence responses, including *OsMAPK12*, *OsMAPK5* and *OsMAPK6*. The results of Cheong *et al.* (2003) and Koo *et al.* (2009) proved that rice *OsMAPK12* is involved in plant disease resistance to several pathogens, including *Phytophthora parasitica*, *Pseudomonas syringae* and *M. oryzae*, whereas, the data of Xiong and Yang (2003) showed the involvement of rice *OsMAPK5* in disease resistance to *M. oryzae.* They generated different transgenic rice plants with overexpression and suppression of *OsMAPK5*, and found that the suppression lines showed enhanced disease resistance to the pathogen, while the overexpression lines showed a similar sensitive reaction as seen in wildtype plants. *OsMAPK6* was found to be a negative regulator for rice resistance to the bacterial blight pathogen *X. oryzae* pv. *oryzae*, the suppression of this gene in rice plants enhanced disease resistance to the bacterial pathogen compared to wildtype plants. The lesion number and bacterial growth decreased significantly, and different sets of defence-responsive genes were induced such as *PR5*, *PR10* and *WRKY30*. Also, they showed that the level of salicylic acid was increased 1.6 fold higher in the *OsMAPK6*-suppression lines compared to wildtype plants (Yuan *et al.*, 2007; Shen *et al.*, 2010).

Table 4.1 Barley full length clones and rice MAPK homologues.

Barley Gene bank accession	Rice homologue	Also Known as	Reference
AK252439	OsMAPK12	BWMK1	He *et al.* (1999)
AK248685	OsMAPK12	BWMK1	He *et al.* (1999)
AK252980	OsMAPK4	OsMAPK2, OsMSRMK3,	Agrawal *et al.* (2003a)
AK250314	OsMAPK11	OsBIMK2, OsMPK2, OsRMAPK2	Liu and Xue (2007)
AK251947	OsMAPK9	-	
AK252802	OsMAPK10	-	
AK251500	OsMAPK14	OsBIMK2, OsMPK2, OsRMAPK2	Hamel *et al.* (2006)

4.2 Barley transformation

The genetic transformation of cereal crops has been widely investigated as a target for crop improvement in the last decades. Different approaches have been applied to introduce transgenes into cereals such as uptake of free DNA into the protoplast of cereal crops, particle gun bombardment and *Agrobacterium tumefaciens* mediated transformation. Regarding *Agrobacterium*-mediated transformation, the delivery of foreign genes into dicots plants has been well established in contrast with monocot plants which are not the natural host for *Agrobacterium*, but this obstacle has been overcome by introducing different super binary vectors which have additional virulence gene copies such as *virB, virC1* and *virG* genes (Klein *et* al, 1988; Shimamoto *et al.*, 1989; Tingay *et al.*, 1997; Goedeke *et al.*, 2007)

In this study, the supervirulent strain of *A. tumefaciens* EHA105 (which contains the Ti plasmid pTiBoS42 with an additional *virG* gene) was used to introduce the transgene *HvMAPK4* into barley plants. In order to do this, the binary vector pWBVec.8 with a hygromycin gene cassette for plant selection was utilised (Fig. 2.1).

For barley transformation and the tissue culture course, the procedure of Bartlett *et al.* (2008) was followed with some modification; particularly the modification was related to the regeneration stage by deletion of the transition step (2 weeks step) and moving directly from the callus induction step to the regeneration stage for six weeks. The second difference was decreasing the hygromycin concentration to 25 mg/l compared to 50 mg/l in Bartlett *et al.* (2008) for selection in the regeneration stage. It is crucial to include the antibiotic (hygromycin) in any type of media for barley tissue culture regime to ensure there is no escape from the selection, so that all of the resulting plants are transformed successfully (Tingay *et al.*, 1997; Bartlett *et al.*,2008; Hensel *et al.*, 2008).

In terms of media composition, the cupric sulfate concentration of 1.25 mg/l was used in callus induction medium and regeneration stage, this concentration is 50 fold higher than the concentration in Murashige and Skoog (MS) salts (0.025 mg/l). The addition of extra amount of cupric sulphate was recommended by several workers because of the concentration of copper in MS medium is not sufficient to support the growth of cereal (Purnhauser, 1991; Dahleen, 1995).

A promotional effect of cupric sulfate on callus growth has been noted in wheat and barley (Purnhauser, 1991; Dahleen, 1995). The elevated concentration of cupric sulfate in callus and regeneration medium led to increase the number of green plants per embryos and the percentage of green plants (Bregitzer *et al.*, 1998). The promoting effect of copper can be attributed to its involvement in several metabolic activities such as protein and carbohydrate metabolism (Joshi and Kothari, 2007), and to its role in blocking ethylene perception (Chang and Shockey, 1999).

The inoculation of immature embryos of barley cultivar Golden Promise with *Agrobacterium* was performed on the same day of embryos preparation; the results of Bartlett *et al.* (2008) showed there was no significant effect in the timing of embryos inoculation when they performed two inoculation times, at the same day of preparation or the day after preparation of immature embryos. Regarding the orientation of the scutellum, the scutellum was placed side down on the CIM after the transfection with *Agrobacterium*, in the paper of Wan and Lemaux (1994) they placed scutellum side-up, but more recently, most workers preferred to place the scutellum side-down on the medium because somatic embryogenesis and plant regeneration are normally initiated at the surface of the scutellum (Lange *et al.*, 2006; Hensel *et al.*, 2008).

4.3 Analysis of transgenic barley plants

The results of three experiments of *Agrobacterium*-mediated barley transformation into immature embryos of cultivar Golden Promise revealed that the transformation with antisense-*HvMAPK4* construct was the most successful experiment and produced 11 independent transgenic lines, while the overexpression-*HvMAPK4* construct was less successful and produced only five independent transgenic lines. Regarding the third experiment eight independent lines were generated with empty vector pWBVec.8 as control.

The analysis of second generation transgenic barley with different transgenes by PCR showed the presence of the *hygromycin* resistant gene (*hpt*) and *HvMAPK4* transgene for both overexpression and antisense constructs with a total percent of transformation efficiency of 9.5%. This percentage transformation efficiency is in good agreement with the results cited in different paper, such as 4.2% in Tingay *et al.* (1997), 12% in Matthews *et al.* (2001).

The transformation efficiency is mainly dependent on several issues, such as *Agrobacterium* strains, donor plants, composition of medium and the binary vectors. The barley genetic background is one of these factors, cultivar Golden Promise which is used in this study to produce stable transformation was used in several previous studies and these works underline the amenability of this cultivar to *Agrobacterium*-mediated transformation (Murray *et al.*, 2004; Lange *et al.*, 2006; Bartlett *et al.*, 2008; Hensel *et al.*, 2008).

The strong constitutive promoter *Act1* from rice was used along with the octopine synthase (*OCS*) as terminator in the binary vector of pWBVec.8 to control the expression of *HvMAPK4* gene in transgenic plants. The analysis of *HvMAPK4* gene expression in different barley lines, including *HvMAPK4*-overexpression and *HvMAPK4*-antisense plants along with wildtype Golden Promise cultivar as a control, showed, as expected, an up-regulation in the level of *HvMAPK4* in overexpressing plants and down-regulation of *HvMAPK4* in the antisense plants at the mRNA level.

All the transgenic lines were monitored to check any abnormalities during the growth; no obvious phenotypical differences between transgenic barley and the wildtype were seen in this study, and all of these transgenic plants were fertile and produced seeds. It's noteworthy that the modification of some MAP kinases in different plants can be led to observable phenotypes such as dwarfism, curled leaves and flowers, reduction in fertility and pollen production which were seen in the *Arabidopsis* mutant of *mpk4*. The dwarfism in the *Arabidopsis* mutant was attributed to the decrease in the cell size (Petersen *et al.*, 2000). Similar results were seen when the two MAPK4-regulating MKKs, *MKK1* and *MKK2* are knocked out (Qiu *et al*, 2008). Additionally, the suppression of *OsMAPK5* led to develop observable brownish stripes on rice leaves at the late vegetative stage (Xiong and Yang, 2003). Shen *et al.* (2010) showed that the overexpression of *OsMAPK6* in rice plants developed brownish lesion on their leaves compared to the wildtype plants.

4.4 The pathogenicity of *M. oryzae* on barley cv. Golden Promise

The pathogenic strain Guy 11 of the hemibiotrophic pathogen *M. oryzae*, which caused blast disease, was selected in this study because of its importance as a serious pathogen on several cereal host families. This pathogen is considered as a model biotrophic fungus for investigating plant-microbe interaction (Gilbert *et al.*, 2006).

Regarding the infection experiment, the *M. oryzae* pathogen was able to stimulate the disease symptoms on barley leaves five days post-inoculation, and the conidial dilution 2 X 10^5 conidia per ml was sufficient to stimulate these symptoms on barley. The disease severity was 4.3 according to the description of Valent *et al.* (1991), while the lesion size was found to be 4.30 mm/leaf. The rapid expansion of the lesions on barley leaves was noticeable in the first five days post inoculation. The disease severity and lesion size on the wildtype indicated the virulence of this pathogen on barley plants.

It is known that this pathogen will grow in a range of temperatures of between 12-32 °C, and requires high humidity and long periods of plant surface wetness to continue the life cycle, and the infection process requires high temperature (26 °C), high humidity and darkness to ease the penetration of the host cells (Kato, 2001; Zellerhoff *et al.*, 2006). All of these conditions were followed in the infection experiments to provide the pathogen with optimal conditions for infection.

HvPR1 is a gene that is characteristically upregulated after infection by biotrophic pathogens as part of the plant defence response. The pathogenesis related proteins are known to be absent or present at low concentration in healthy tissues, but these proteins were found to be increased significantly in response to infection (van Loon *et al.*, 2006). The expression of *HvPR1* was investigated in wildtype barley leaves after infection with *M. oryzae*, and *HvPR1* expression was enhanced as a response to the pathogen infection at 24 and 48 h post-inoculation. The *HvPR1* was undetectable in healthy tissue, but the *HvPR1* was highly expressed at 24 h post-inoculation.

The up-regulation of barley PR1 protein in fungal inoculated plants is consistent with the results of Agrawal *et al.* (2001) when they reported that both *OsPR1a* and *OsPR1b* transcripts reached a maximum at 48 h post-inoculation in rice and *M. oryzae* interaction, and no detection was seen for either gene in healthy rice seedling leaves.

The primary reason to check for *HvPR1* accumulation in barley leaves after challenging with the hemibiotrophic pathogen is the use of this pathogenesis related protein family as a marker for systemic acquired resistance, and the induction of PR1 protein is one of the best characterized defence responses during plant-microbe interaction. In addition, several studies revealed the association between PR1 accumulation and disease resistance in different host-microbe interaction such as tobacco plants and the fungal pathogens *Peronospora tabacina* and *Phytophthora parasitica* var. *nicotinae*, and

tomato plant and *P. infestans* (Alexander *et al.*, 1993; Niderman *et al.*, 1995). The biological function of PR1 protein is still unclear, but it was found that the PR1 protein and other pathogenesis related proteins such as β-1, 3-glucanase (PR2), chitinase (PR3), PR4 and osmotin had a strong antimicrobial activity *in vitro* (Buchel and Linthorst, 1999; Honée, 1999).

RNA blot analysis of *HvMAPK4* in response to the infection showed an induction in the level of expression at 24 and 48 h post-inoculation compared to time 0, this result is consistent with the reported induction of the homologue *OsMAPK4* in rice leaves after infection with the blast pathogen. Apart from *OsMAPK4*, expression of other rice MAPKs was also showed to be induced as a response to *M. oryzae* infection including *OsMAPK5, OsMAPK8* and *OsMAPK13* in rice plants (Reyna and Yang, 2006).

4.5 Phytohormone measurements

To explore the biological function of barley *HvMAPK4* in the barley response to the hemibiotrophic pathogen *M. oryzae*, *HvMAPK4*-antisense, *HvMAPK4*-overexpression transgenic plants and wildtype plants were infected with the pathogen and the change in different plant hormone status were measured as detailed below.

4.5.1 Salicylic acid level

It has been reported that salicylic acid is involved as a signal molecule in mediating stress response, in particular localised defence and systemic acquired resistance (Dempsey and Klessig, 1995). Enhanced accumulation of endogenous salicylic acid has been noticed in different plants as a response to plant pathogens (Malamy *et al.*, 1990; Uknes *et al*., 1992).

The results described in 3.7.1 showed that the barley wildtype Golden Promise accumulated up to 10 fold higher level of salicylic acid at 48 h post-inoculation compared to the base level in healthy tissue. This is consistent with the increase in *HvPR1* gene expression in wildtype at 24 and 48 h post-inoculation (Fig. 3.19) as increases in endogenous salicylic acid is thought to be the driver for *PR1* gene expression after challenging with *M. oryzae*. This conclusion is in accordance with several papers that show the increase in the level of endogenous salicylic acid in pathogen inoculated plants coincides with accumulated expression of genes encoding

pathogenesis-related proteins (PR) (Gaffney *et al.*, 1993; Delaney *et al.*, 1994; Agrawal *et al.*, 2001; Xiong and Yang, 2003; Park *et al.*, 2009; Shen *et al.*, 2010).

For the transgenic plants, the *HvMAPK4*-antisense lines produced elevated level of endogenous salicylic acid as a response to the infection with *M. oryzae* 1.6 fold higher than the level in *HvMAPK4*-overexpression and wildtype plants. This elevation was obvious at 24 h post-inoculation and even higher at 48 h post-inoculation. Thus, the *HvMAPK4* seems to be acting as a negative regulator for salicylic acid accumulation after infection. An important point to note is that salicylic acid levels are not enhanced in the unchallenged antisense plants (Fig. 3.20), thus *HvMPK4* at basal levels is not acting as a constitutive salicylic acid regulator.

Previous studies have showed that the suppression of different MAPKs in rice and *Arabidopsis* plants resulted in high accumulation of endogenous salicylic acid, even in the absence of pathogen attack; such a finding was reported in rice studies related to the bacterial pathogen *Xanthomanas oryzae,* the causal organism of bacterial streak disease. The suppression of *OsMAPK6* in rice plant enhanced the accumulation of salicylic acid up to 17 µg/g FW compared to the levels in wildtype plants which was 8.5 µg/g FW. Thus it was concluded that *OsMAPK6* functions as a negative regulator in rice resistance to the biotrophic pathogen *X. oryzae* (Yuan *et al.*, 2007; Shen *et al.*, 2010).

In *Arabidopsis* plants, the loss of *MAPK4* function leads to an increase in the endogenous level of salicylic acid. Salicylic acid levels were found to be higher 9 fold in the *mpk4* mutant compared to wildtype, the level of salicylic acid was 1300 ng/g FW in *mpk4* mutant compared with 150 ng/g FW in wildtype. *mpk4* showed enhanced disease resistance to different biotrophic pathogens such as *P. syringae* pv. *tomato* strain DC3000 and oomycete pathogen *Hyaloperonospora parasitica* (Petersen *et al.*, 2000). The oomycete pathogen was virulence and rapidly colonized and induced disease symptoms in the wildtype compared with *Arabidopsis mpk4* mutant. It's noteworthy that these *Arabidopsis mpk4* mutants exhibit dwarfism and leaf curling abnormalities compared to the wildtype of *Arabidopsis*. This is also seen when the two MAPK4-regulating MKKs, *MKK1* and *MKK2* are knocked out (Qiu *et al*, 2008).

4.5.2 Jasmonic acid level

Jasmonic acid has been studied by many scientists in its role as a signalling molecules activating different gene expression in the response to mechanical wounding, environmental stresses and pathogen attack (León and Sánchez-Serrano, 1999). In the barley- *M. oryzae* interaction, it was found here that the levels of jasmonic acid in the wildtype plant were constant both pre and post-inoculation with this pathogen. The amount of jasmonic acid in healthy tissue was 8.72 ng/g, and was 8.77 ng/g at 48 h post-inoculation with *M. oryzae* (Fig. 3.21). Statistical analysis showed there was no significant difference between the wildtype and antisense-*HvMAPK4* plants in the level of jasmonic acid pre and post-inoculation.

Current thinking is that the role of jasmonic acid in plant disease is mainly dependent on the life style of the pathogen, e.g. in *Arabidopsis*, the blocking of jasmonic acid enhanced the disease susceptibility to necrotrophic pathogens such as *A. brassicicola* and *B. cinerea*, but not to the biotrophic pathogen *Peronospora parasitica* (Thomma *et al.*, 1998).

The potential of jasmonic acid to provide different host plants with resistance against the attack by different pathogens has been reported in several plant-necrotrophic pathogens interactions such as potato and *P. infestans*; *Arabidopsis* and *B. cinerea* and tomato to *B. cinerea* (Cohen *et al.*, 1993; Thomma *et al.*, 1998; Yu *et al.*, 2009).

In wheat plants, it was found that the pathogenic effect of crown rot pathogen *Fusarium pseudograminearum* was decreased after the treatment of wheat cultivars with methyl jasmonate, and different defence genes were found to be induced rapidly after this treatment including several PR proteins (Desmond *et al.*, 2006), these results suggest the significant role of jasmonic acid in monocots defence against necrotrophic pathogens.

In the experiments reported here, the overexpression of barley *HvMAPK4* gives rise to constitutively enhanced levels of jasmonic acid in transgenic barley, up to 2 fold higher than the levels in the wildtype and *HvMAPK4*-antisense plants. This result suggests that the barley *HvMAPK4* is a positive regulator for jasmonic acid accumulation, even in the absence of biotic stress. Similar results were obtained in the study of Shen *et al.* (2010) when they generated transgenic rice with modification in *OsMAPK6* gene; they reported

that the overexpression of this gene enhanced the level of jasmonic acid up to 2 fold more over than what was seen in wildtype and suppression *OsMAPK6* lines.

4.5.3 Ethylene production

The participation of the plant hormone ethylene in the regulation of several plant responses to biotic and abiotic stresses was reported in many studies (Hoffman *et al.*, 1999; Fan *et al.*, 2000; Shinshi, 2008), but the role of ethylene in the plant response to the pathogen attack has been a matter of some debate. Several papers revealed that the plant hormone ethylene is important for infection and disease development of necrotrophic pathogens rather than biotrophic pathogens (Steven *et al.*, 1998; Hoffman *et al.*, 1999; Thomma *et al.*, 1999).

Jasmonic acid and ethylene can act synergistically in plant regulation of disease resistance to several types of pathogen attack (Turner *et al.*, 2002); it was reported that the treatment of tomato fruits with methyl jasmonate decreased the disease severity of *B. cinerea* , and this decrease was accompanied with elevated levels of ethylene in tomato (Yu *et al.*, 2009).

The results from this study showed that the overexpression of *HvMAPK4* gene in barley plants produced higher amount of ethylene (up to 1.7 fold) at 48 h post-inoculation with *M. oryzae* compared to the wildtype and *HvMAPK4*-antisense plants (Fig. 3.22), whereas, no significant difference was seen between wildtype and *HvMAPK4*-antisense plants. Thus, the *HvMAPK4* gene in barley appears to act as a positive regulator of biotic stress induced ethylene. In view of the enhanced levels of jasmonic acid in the overexpression lines and the known synergy of action between ethylene and jasmonic acid, it appears that *HvMAPK4* is a positive determinant of the plant wound and necrotrophic stress response.

4.6 Hydrogen peroxide and catalase activity

The data generated in this study showed that barley with different levels of *HvMAPK4* gene expression performed in different ways in their production of hydrogen peroxide as a response to the infection with *M. oryzae*. The *HvMAPK4*-antisense lines showed an increase in the level of hydrogen peroxide up to 1.5 fold that of the wildtype, whereas, a significant reduction was seen in the *HvMAPK4*-overexpression lines compared to the wildtype Golden Promise at 24 and 48 h post-inoculation (Fig. 3.23). This finding

indicates that barley *HvMAPK4* gene appears to be a negative regulator of biotic stress-induced hydrogen peroxide.

The early stage of the plant response to different pathogens is associated with hydrogen peroxide production. The role of hydrogen peroxide varies from the direct toxic effect on the pathogen itself by inhibition of the growth and development of pathogen, or by reinforcing the cell wall by different processes such as cell wall lignifications, lignin is considered as the second abundant polymer after cellulose with a pivotal role in providing the plant with structural support, and hydrogen peroxide is involved in the final step of oxidative polymerization to produce lignin (Kovácik *et al.*, 2010). Hydrogen peroxide also contributes to cross-linking of proline rich protein and other cell wall polymers to restrict the penetration of plant pathogen and to inhibit the spreading of pathogen toxins to plant cells (Kuzniak and Urbanek, 2000; Lin *et al.*, 2005; Shetty *et al.*, 2007). Additionally, hydrogen peroxide can also acts as a messenger to stimulate the expression of several genes involved in plant resistance such as the *PR1* gene (Lin *et al.*, 2005).

The analysis of catalase activity showed that the *HvMAPK4*-overexpression lines responded to the pathogen by increases in catalase activity (Fig. 3.24). This increase reached a maximum at 48 h post-inoculation and was 2.7 fold higher that in the *HvMAPK4*-antisense lines which showed a reduced activity for catalase relative to the controls. Catalase is one of the most abundant detoxifying enzymes with significant role in the control of ROS production and accumulation in plant (Asada, 1999).

Collectively, the *HvMAPK4*-overexpression lines showed an increase in catalase activity and a significant reduction in hydrogen peroxide at the same time. The catalase enzyme converts hydrogen peroxide to H_2O and O_2, thus, any increase in the activity of catalase more likely will lead to a decrease in the hydrogen peroxide levels. The opposite was found in the *HvMAPK4*-antisense lines, which showed significant suppression for catalase activity and elevated level of hydrogen peroxide at 48 h post-inoculation with *M. oryzae*.

4.7 Disease severity in transgenic and wildtype of barley plants

The antisense suppression of *HvMAPK4* gene in barley plants reduced the level of sensitivity to the pathogen *M. oryzae* compared to the wildtype plant and *HvMAPK4*-overexpression lines. All of the disease parameters including lesion size, lesion number and disease severity in *HvMAPK4*-antisense were significantly difference than in both wildtype and *HvMAPK4*-overexpression lines.

Furthermore, *HvPR1* was highly expressed in *HvMAPK4*-antisense lines, with a detectable signal prior to infection and with a strong accumulation after infection as compared to the wildtype and the *HvMAPK4*-overexpression plant. This suggests that barley *HvMAPK4* gene negatively regulates the expression of the *HvPR1* gene. However the *HvMAPK4*-overexpression plants also showed enhanced levels of *HvPR1* prior to infection, it is possible that this is caused by the constitutively high levels of jasmonic acid in these plants as although *PR1* gene expression is usually associated with salicylic acid, expression of this gene in rice is also known to be enhanced by jasmonic acid (Agarwal *et al*, 2000).

All of these findings indicate that the *HvMAPK4* gene acts as a negative regulator for barley resistance to hemibiotrophic pathogens such as *M. oryzae*. In *Arabidopsis,* the loss of *MAPK4* functions leads to increase the resistance level to several biotrophic pathogens including the bacterial pathogen *Pseudomonas syringae* pv. *tomato* and the fungal pathogen *Peronospora parasitica*, also, the expression level of pathogenesis-related proteins including *PR1*, *PR2* and *PR5* were expressed constitutively in *mpk4* plants compared to wildtype (Petersen *et al.*, 2000). Similar results have been found for other cereal MAP kinase genes, for example in rice, the suppression of *OsMAPK5* gene expression also led to enhanced disease resistance to the *M. oryzae*. This resistance was accompanied with strong expression for pathogenesis-related proteins *PR1* and *PR10*. The opposite phenotype was found in overexpression of *OsMAPK5* gene in rice, which showed a higher sensitivity level to the same pathogen (Xiong and Yang, 2003).

It has been found that the rice *OsMAPK6* gene is a negative regulator for disease resistance toward the biotrophic bacterial pathogen *X. oryzae* pv. *oryzae*. The suppression of *OsMAPK6* gene in rice led to enhanced disease resistance to this bacterial pathogen, the suppression of this gene in rice plants led to increase the level of salicylic acid and induced the expression of rice *PR1* gene (Shen *et al.*, 2010). It is

probable therefore that multiple MAP kinases operate to negatively regulate the cereal salicylic acid mediated basal immune system.

4.8 Signalling in the response of barley to *M. oryzae* infection

The plant response to pathogens is a complex process with involvement of several phytohormones, predominantly salicylic acid, jasmonic acid and ethylene. Different signalling pathways regulate plant responses to pathogen, and these responses can be classified into two categories, resistance or susceptible reaction. The resistance reaction to the plant pathogen is depend on several factors including the speed of defence processes starting with recognition of potential pathogens, transport and secretion of defence compounds through PAMP-triggered immunity (see 1.5.2), while any failure in the recognition and response to the pathogens leads to a susceptible reaction. A successful pathogen needs to suppress plant defences in order to establish disease (O'Donnell *et al.*, 2003; Gomez-Gomez and Boller, 2000).

The data produced in this project has shown that the suppression of barley *HvMAPK4* gene enhances disease resistance to the hemibiotrophic pathogen *M. oryzae*. Transgenic lines of suppression *HvMAPK4* gene were more resistant to blast pathogen compared to wildtype and overexpression-*HvMAPK4* lines. It is apparent from these results that barley plant responded to the blast pathogen via the salicylic acid pathway and not the jasmonic acid/ ethylene pathway. This finding is in agreement with the concept that the resistant reaction to biotrophic pathogen requires salicylic acid, while the response to necrotrophic pathogen requires jasmonic acid and ethylene (Feys and Parker, 2000; Petersen *et al.*, 2000).

Elevated levels of salicylic acid were found in *HvMAPK4* suppression lines, and this elevation was accompanied with an increase in the level of hydrogen peroxide and a reduction in catalase activity. The inhibition of catalase activity in *HvMAPK4* suppression lines can be attributed to the elevated level of salicylic acid, because it has been reported that salicylic acid is a strong inhibitor of catalase and other scavenging enzymes (Lamb and Dixon, 1997). It was found that the injection of high concentration of salicylic acid into plants gives an increase in the level of hydrogen peroxide (Chen *et al.*, 1993). Also, the accumulation of hydrogen peroxide has been demonstrated by several authors as a response to salicylic acid application, proposing that hydrogen

peroxide is a downstream signal of salicylic acid during systemic acquired resistance (Rao *et al.*, 1997; Shirasu *et al.*, 1997; Harfouche *et al.*, 2008).

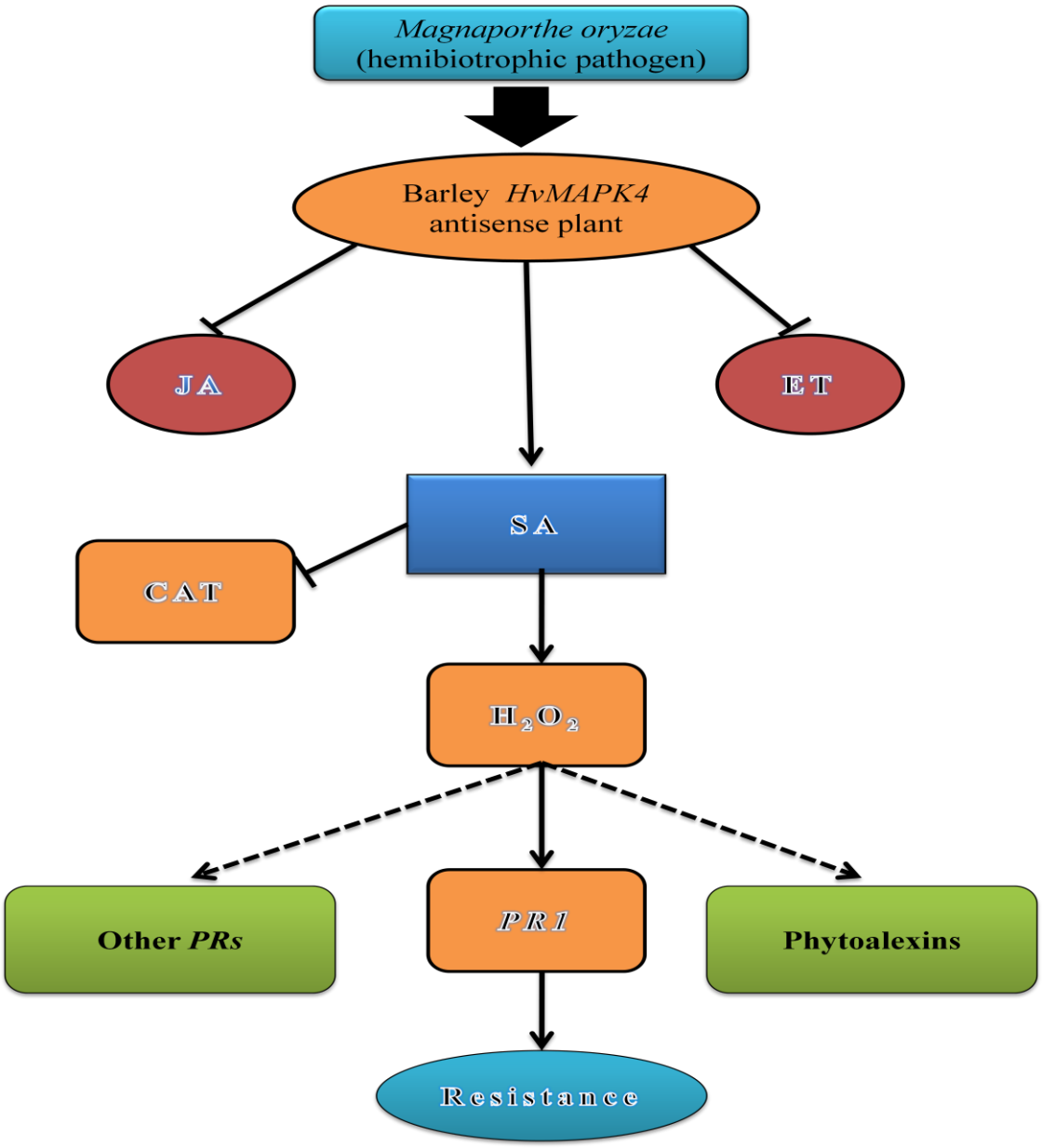
It's also believed that the hydrogen peroxide is an upstream signal of salicylic acid during oxidative burst and induced resistance. The results of Leon *et al.* (1995) showed that the infiltration treatment of tobacco leaves with hydrogen peroxide at concentration 300 mM leads to induces the accumulation of salicylic acid and benzoic acid. Furthermore, they showed that this infiltration treatment leads to a rapid activation of benzoic acid 2-hydroxylase (B2H) which is responsible o salicylic acid formation from benzoic acid. Similar findings were reported in the study of Chmnongpol *et al.* (1998) when they concluded that the hydrogen peroxide is an upstream signal of salicylic acid in tobacco plants and a strong inducing agent of systemic acquired resistance.

Regarding jasmonic acid and ethylene, the results show that overexpression of *HvMAPK4* gene leads to a constitutive increase in the production of jasmonic acid and ethylene compared to wildtype and *HvMAPK4*-antisense lines. Different mutants in *Arabidopsis* which impair jasmonic acid production (*fatty acid desaturase*: *fad3, fad7, fad8* triple mutants) or jasmonic acid perception (*cornatine insensitivity 1: coi1* and *jasmonic acid resistant1: jar1*) showed enhanced level of sensitivity to wide range of necrotrophic pathogens including *A. brassicicola*; *B. cinerea*; *Pythium irregulare* and *Erwinia carotovora* (Staswick *et al.*, 1998; Thomma *et al.*, 1998; Norman-Setterblad *et al.*, 2000; Stintzi *et al.*, 2001). This it can be predicted that the *HvMAPK4*-overexpression lines may show some constitutive protection against necrotrophic pathogens.

The increase in jasmonic acid level in *HvMAPK4*-overexpression lines was accompanied with an increase in ethylene production compared to the wildtype. Many studies have now shown that ethylene and jasmonic acid act in concert to defend the plant against necrotrophs or feeding insects (Turner *et al.*, 2002; Shinshi, 2008; Yu *et al.*, 2009). Jasmonic acid and ethylene together are essential for the expression of different responsive genes in *Arabidopsis* and *A. brassicicola* interactions including defence-related gene *PDF1.2* and basic chitinase (*B-CH*) (Penninckx *et al.*, 1996; Shinshi, 2008). The exogenous application of ethylene and jasmonic acid together synergistically enhance *PDF1.2* expression and that of other defensive genes in *Arabidopsis* (Penninckx *et al.*, 1998).

The evidence of antagonistic effect of salicylic acid and jasmonic acid signalling was provided by several papers (Lee *et al.*, 2004; Bostock, 2005; Bruce and Pickett, 2007). In *Arabidopsis*, Peterson *et al.* (2000) reported that *MAPK4* is essential for induction of jasmonic acid responsive genes including *PDF1.2*, while the loss of MAPK4 function in *Arabidopsis* mutants constitutively expressed salicylic acid responsive *PR* genes, they concluded that this gene repressed salicylic acid biosynthesis and promoted jasmonic acid biosynthesis, suggesting its function as a regulator for negative cross talk between salicylic acid and jasmonic acid biosynthesis.

The up-regulation of *HvPR1* in *HvMAPK4*-antisense lines can be explained by the role of salicylic acid and hydrogen peroxide, which were elevated in the suppression lines. This idea is in good agreement with the results of Agrawal *et al.* (2001) who showed that the treatment of rice leaves with both salicylic acid and hydrogen peroxide enhanced the expression of *OsPR1* gene. This expression was increased in abundance at 24 h and 48 h after treatment. Additionally, Iwai *et al.* (2004) showed that the elevated level of salicylic acid in rice leaves was accompanied with an increase in the expression level of *OsPR1* as a response to the challenge with blast pathogen *M. oryzae*. A model for the signalling pathways in barley in response to *M. oryzae* infection is illustrated in Fig. 4.1.



SA: Salicylic acid
JA: Jasmonic acid
ET: Ethylene
CAT: Catalase activity
H_2O_2: Hydrogen peroxide
PR1: pathogenesis-related protein 1

Fig. 4.1 Module for signalling in the response of barley *HvMAPK4* antisense plant to *M. oryzae* infection.

4.9 The response of *HvMAPK12*- antisense transgenic lines to the infection with *M. oryzae*

The *HvMAPK12* gene was isolated and identified in rice, and belongs to MAP kinase group D with TDY phosphorylation motif (He *et al.*, 1999; Agrawal *et al.*, 2003b). Transgenic barley plants that down-regulated *HvMAPK12* by antisense were also analysed as a further control. Statistical analysis of disease symptoms showed that there was no significant difference between *HvMAPK12*-antisense transgenic lines, barley bearing the empty vector pWBVec.8 and the wildtype in their response to the infection with *M. oryzae*. All of these backgrounds were sensitive in the same degree to the pathogen in terms of disease severity, lesion size and number. Similar results were seen for ethylene and hydrogen peroxide production at time 0 and 24 h post-inoculation. This indicates that the suppression of *HvMAPK12* had no significant effect on barley response to the pathogen, and thus the effect seen in the *HvMAPK12* lines here is specific to this gene.

Previous workers have found that the overexpression of *HvMAPK12* gene in tobacco plants under the control of cauliflower mosaic virus promoter (35S) showed elevated expression levels for several *PR* genes including *PR1*, *PR2*, *PR3*, *PR4* and *PR5* after the inoculation with *Phytophthora parasitica* var *nicotianae* and *Pseudomonas syringae* pv. *tabacci*. Additionally, these transgenic lines exhibited enhanced resistance to each pathogen compared to the wildtype (Cheong *et al.*, 2003). Similar results were reported in generated transgenic rice with overexpression of *HvMAPK12*; these transgenic lines exhibited enhanced resistance to the blast pathogen (*M. oryzae*) and showed elevated level of salicylic acid and hydrogen peroxide up to 5.5 and 5 fold, respectively, higher than in wildtype (Koo *et al.*, 2009).

To summarise, our data showing no significant difference in barley susceptibility to the blast pathogen between both wildtype and *HvMAPK12* suppression lines, and the results of Koo *et al.* (2009) with overexpression- *HvMAPK12* in rice which showed enhanced resistance, these findings suggest that *HvMAPK12* gene is a positive regulator of barley systemic resistance to blast pathogen. Thus it would appear that *HvMAPK12* may function in an opposing manner to *HvMAPK4*, and the two kinases may represent a reciprocal mechanism in which different MAP kinases either negatively or positively regulate the plant response to biotic stress.

4.10 The effect of exogenous salicylic acid and hydrogen peroxide on *in vitro* growth of *M. oryzae*.

As shown in the results chapter (3.11), the exogenous application of salicylic acid and hydrogen peroxide had a significant influence on *in vitro* growth of *M. oryzae*. Both mycelium growth and dry weight were inhibited completely at a salicylic acid concentration of 435 mM and hydrogen peroxide concentration of 50 mM. The results also proved the ability of this pathogen to grow at concentrations of salicylic acid up to 290 mM and hydrogen peroxide concentrations of 10 mM.

The antifungal activity of salicylic acid found here is in good agreement with several studies, which showed that salicylic acid was effective in inhibiting the growth of different fungal pathogens including *Penicillium expansum*, *Alternaria alternata* and *Fusarium oxysporum* f.sp *niveum* (Qin *et al.*, 2003; Wu *et al.*, 2008). Regarding hydrogen peroxide, the data is consistent with the findings of Shetty *et al.* (2007) in which they showed that the hemibiotrophic pathogen *Septoria tritici* was tolerant to hydrogen peroxide concentration of 5 mM, but this tolerance decreased significantly at high concentrations reached 10 and 50 mM.

Comparing to the maximum concentrations for both salicylic acid and hydrogen peroxide which found in barley leaves (see 3.7.1 and 3.78 for salicylic acid and hydrogen peroxide, respectively), the role of salicylic acid and hydrogen peroxide in barley resistance to the blast pathogen *M. oryzae* seems to be involved in activation of different sets of defensive genes rather than the direct antimicrobial activity, which has been shown in this experiment.

4.11 Summary of the analysis of *HvMAPK4.*

In summary, the results shown here indicate that the barley *HvMAPK4* gene plays a pivotal role in barley- blast pathogen (*M. oryzae*) interaction. The down regulation of *HvMAPK4* gene expression in barley leads to an increase the level of resistance, while the overexpression of *HvMAPK4* gene has no significant effect on the pathogenecity of *M. oryzae*. Furthermore, the suppression of *HvMAPK4* gene in barley plants increased the level of salicylic acid and hydrogen peroxide, and decreased the catalase activity. Additionally, the suppression of *HvMAPK4* gene expression in barley resulted in a constitutive increase in the expression of pathogenesis-related protein. The opposite was found in *HvMAPK4* overexpression plants, which produced lower amounts of salicylic acid and hydrogen peroxide after infection and showed increased activity for catalase compared to *HvMAPK4*-antisense plants.

The overexpression of the *HvMAPK4* gene in barley also resulted in a constitutive accumulation for jasmonic acid and increased production for ethylene compared to wildtype plant and suppression *HvMAPK4* plant. These results suggest that the barley *HvMAPK4* behaves as a positive regulator for jasmonic acid accumulation and ethylene production even in the absence of biotic stress.

The present results also proved the inhibitory effect of both salicylic acid and hydrogen peroxide on *in vitro* growth of *M. oryzae*.

4.12 Future work

For future work, several experiments can be performed to clarify the role of barley *HvMAPK4* gene in biotic and abiotic stress responses as:

1-The response of *HvMAPK4*-overexpression and antisense barley plants to the infection with the necrotrophic fungal pathogen *B. cinerea.*

2-The effect of the *HvMAPK4* gene modification on the barley tolerance to the drought and salt stresses.

Additionally, different experiments can be carried out to detect the activity of barley HvMAPK4 protein as:

1-Immunoblot analysis of barley HvMAPK4 protein in different barley backgrounds (*HvMAPK4-o*verexpression, antisense and wildtype plants) by using anti-HvMAPK4 antibody to detect the level of HvMAPK4 protein.

2-Detection of barley HvMAPK4 kinase activity in different barley backgrounds (*HvMAPK4*-overexpression, antisense and wildtype plants) by using kinase in-gel activity and MBP as a substrate.

APPENDIX

5.1 Full length cDNA barley clone

Genebank accession AK252980 (*HvMAPK4*)

GAGGCCCCAGTGGAGCTACGTACGTACGTACGTTCCTTCCCCCGGAATAAAAAAGCCTTCCTGCT
65

CGGTCGCTCTCCGCCTTTGCCTCTGCCTTCGTATCCCTCATCCCCTCCCTCCCCCGCCGCTCCCG
130

GACGTGCTCTGCTTCTCCAGATCCGCCGCCGACGACGCCGCCGCTGCTGCGTCCCGAGAAAATGG
195

CGATGATGGTGGATCCTCCGAACGGCATCGGAAACCATGGCAAGCACTACTACACCATGTGGCAG
260

ACCATGTTCGAGATCGACACCAAGTACGTGCCCATCAAGCCCATCGGGAGGGGAGCCTACGGGAT
325

AGTTTGCTCCTCCATCAACCAGGAGACCAATGAGAAGGTCGCCATCAAAAAGATAAACAACGTCT
390

TTGACAACCGTGTGGATGCGTTAAGGACGCTGCGCGAGCTGAAGCTCCTCCGGCACCTGCGCCAC
455

GAGAATGTCATTGCTTTGAAGGATATAATGATGCCGATACATAGGAGGAGCTTCAAGGATGTCTA
520

CTTGGTCTCCGAGCTCATGGACACGGATCTGCATCAGATTGTCAAGTCGTCTCAGCCGCTGTCCA
585

ATGACCACTGCCAGTATTTCCTTTTTCAGCTGCTCCGAGGACTGAAGTACCTTCATTCAGCAGGG
650

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715

TGACTTTGGTCTGGCTCGCACAAATAACACTAAAGGTCAGTTTATGACTGAATATGTTGTCACCC
780

GCTGGTATAGAGCTCCCGAGTTGCTGCTCTGCTGCGACAACTATGGCACATCCATAGATGTCTGG
845

TCTGTTGGCTGCATCTTTGCTGAGCTACTTGGCCGCAAGCCGATCTTTCCAGGAACCGAGTGCCT
910

TAATCAGCTTAAGCTTATAGTCAATGTTCTTGGCACCATGAGCGAAGCTGACCTCGCATTCATTG
975

ACAACTCAAAAGCACGCAAGTACATTAAATCCCTTCCATACACCCCAGGGATTCCCCTCAGTAGC
1040

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1105

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1170

CAAACCCTCCTGCTCAGGTGCCCATCGATCTTGACATAGATGAAAACATTGGCACAGATATGATC
1235

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1300

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1365

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1430

AAACGGCGTATGGATTTCTCTAGCTGTGGGTCAGTACTTGGTAGTATATATGGACTACTGTATTG
1495

TAGAAGCGGAGTTCAGTTTATTAAGACTTCGGTAATGAAGAAGCTTGTGTAAGTTGTAATCTTCT
1560

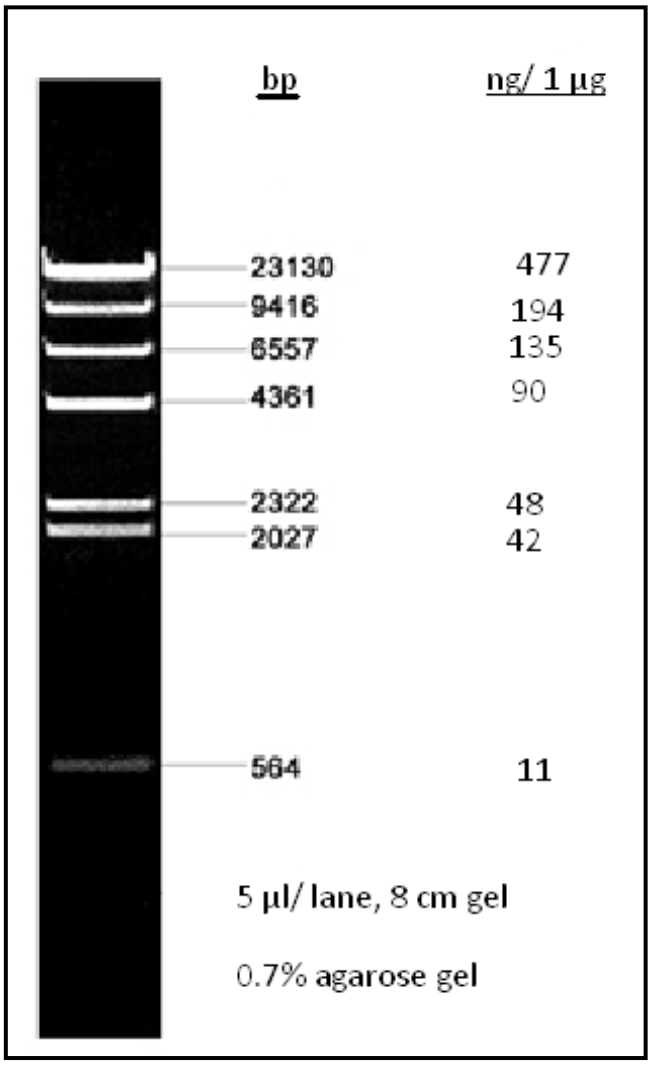
TTTTTCCCCTGATTGTAAGTTGTAATTCTAGACGCTGCTCTATGTATATGAACTACTGTGTTGTA
1625

GAAGAGGAATCCGGTTTATTAAGAATTGGGTTATGAAGAAGCCTGTGTAAGTTGTAATCTTCTCT
1690

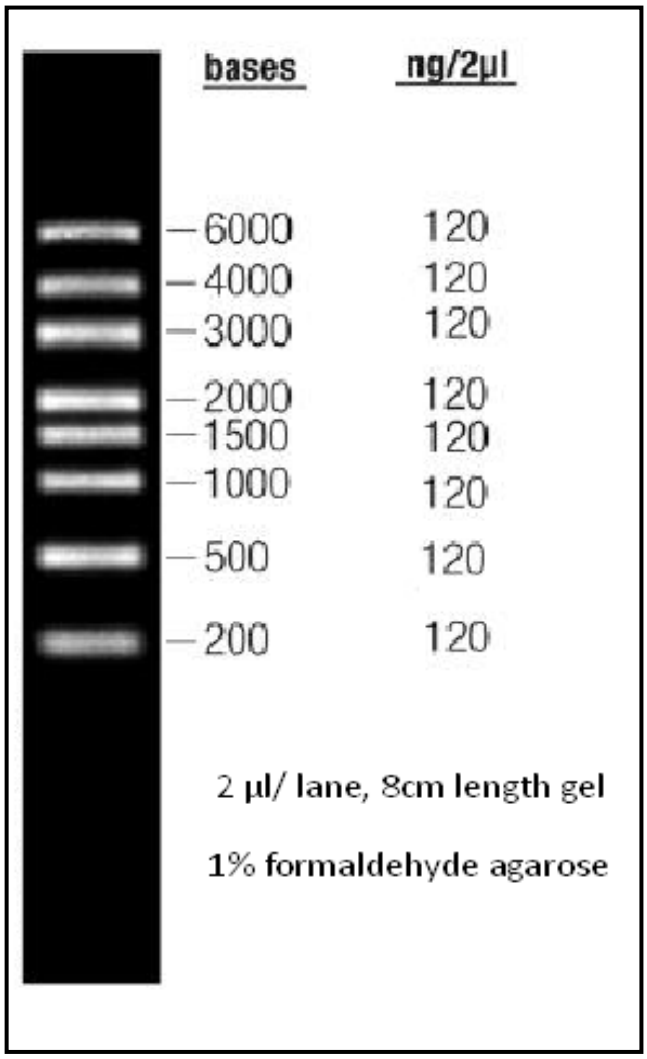
TTTCCCTCTGTAAGTTGTAATTTTAGAATCCGCCCTGTATATGGGGGTCTAAACTATTAAGTGAC
1755

CATGTTTACTTCTAAAAAAAAAAAAAAAA 1784

5.2 Lambda DNA *Hind*III marker (Fermentas)



5.3 RiboRuler high range RNA ladder (Fermentas)



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